

EFFECTS OF CYTOTOXIC DRUGS AND 'ELEMENTAL' DIETS
ON ABSORPTION FROM THE SMALL INTESTINE

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DECLARATION

I declare that this thesis was composed by myself and that the work described was carried out by myself with the following exceptions.

The study described in Chapter 6 was carried out in collaboration with Dr. M.L.G. Gardner and with the technical assistance of Mrs. A. Pryde.

The study described in Chapter 7 was carried out in collaboration with Dr. M.L.G. Gardner.

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12.3.1981

ABSTRACT

Cancer chemotherapy with 5-fluorouracil is often limited by severe gastrointestinal toxicity. The aims of this study were to dissociate the effects of 5-fluorouracil on the intestine from those simply due to reduced food intake and to investigate means of reducing the intestinal toxicity of the drug.

Established methods for the estimation of intestinal water absorption, mucosal DNA contents and cytoplasmic peptide hydrolase activities, with appropriate modifications and improvements where necessary, were validated. During the estimation of absorption in vitro peptidase activities were released from the perfused intestine. The amounts released were similar to those released from intestines perfused in vivo provided that the intestine was removed from an anaesthetized animal; however, they were markedly increased when the rat was killed prior to establishment of perfusion. The importance of this observation to the use of intestinal preparations in vitro and to the physiological site of peptide hydrolysis is discussed. The release of enzyme activities may be increased by anoxia but an attempt to use enzyme measurements for the diagnosis of intestinal ischaemia was unsuccessful.

Intestinal absorption and mucosal DNA contents were severely reduced 3 days after 5-fluorouracil administration. The reduced food intake alone resulted in a much smaller reduction. Also, the Tris and sodium salts of 5-fluorouracil have markedly different systemic toxicities and effects on food intake but similar effects on the intestine. Hence the intestinal effects of 5-fluorouracil cannot be attributed only to a reduced food intake.

The plasma pharmacokinetics of the two salt forms of 5-fluorouracil differ after intraperitoneal injection. This is evidence of a difference in the properties of the two salts and hence it is a property of the Tris salt per se that is responsible for the mortality associated with administration of this salt of the drug.

Intestinal peptide hydrolase activities are reduced after 5-fluorouracil administration and this might impair the digestion and absorption of protein. However, 'elemental' diets do not protect the intestine from the toxic effects of 5-fluorouracil and may even increase the toxicity of the drug.

The intestinal toxicity of 5-fluorouracil varies with the time of drug administration. Least toxicity was observed after injection at 02:00 and maximal toxicity after injection at 15:00. These times coincide with the peak and nadir respectively of the mucosal DNA content and it is suggested that the intestinal toxicity may be related to the proliferative activity of the intestine at the time of injection; this in turn may be related to the feeding pattern of the animals. The possible application of these observations to the clinical use of 5-fluorouracil is discussed.

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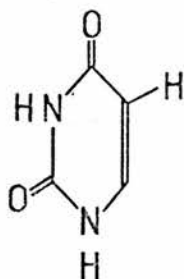
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CHAPTER 1

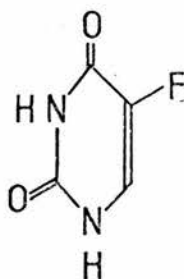
General Introduction

Chemotherapy was introduced in the mid 1950's for the treatment of cancer and, together with radiotherapy, offers great potential for the non-surgical palliation, or even cure, of some cancers. Nevertheless, the success rate of treatment is still low and a great deal of effort is being spent on attempts to improve therapeutic regimes of existing and of new drugs.

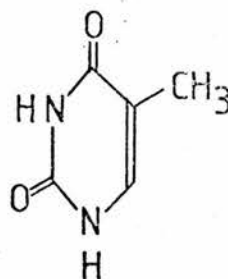
The pyrimidine 5-fluorouracil is an antimetabolite used in the chemotherapy of certain epithelial tumours. It is a structural analogue of thymine (see below) and is thought to prevent cell division by inhibition of thymidilate synthase; an enzyme important in the production of thymine nucleotides for DNA synthesis (Heidelberger et al, 1959). Although 5-fluorouracil is also incorporated into RNA in place of uracil this is not thought to be important in the antitumour activity of the drug (Heidelberger et al, 1960a and b).



Uracil



5-Fluorouracil



Thymine

While surgery and irradiation can be effective in the treatment of localised tumours, the introduction of chemotherapy resulted in a major advance in the treatment of disseminated cancers and by this

method alone a normal life expectancy has been achieved for at least half the patients with ten different types of cancer (Zubrod, 1972). 5-Fluorouracil is most useful in the treatment of carcinoma of the breast (Ansfield et al, 1969) and of the gastrointestinal tract (Hall, 1973; Haskell, 1980, p. 88; Horton et al, 1974; Ratner et al, 1972).

The mechanism of action of the majority of cancer chemotherapeutic agents is either by inhibition of DNA synthesis, e.g. methotrexate, cytosine arabinoside and 5-fluorouracil, or by inhibition of mitosis, e.g. vincristine and vinblastin (see Haskell, 1980, p. 28). They are therefore most active against rapidly growing tumours, in which most of the cells will be dividing at any given time, and have very little activity against slow growing tumours (Zubrod, 1972). However, as is clear from the mechanism of action of these drugs, they are not specific for tumour cells and their use is accompanied by toxic side effects. These are especially noticeable in rapidly proliferating tissues such as the gastrointestinal epithelium and the bone marrow (Haskell, 1980, p. 29; Calabresi and Parks, 1975). Hence, chemotherapeutic agents are only of clinical use if there is a quantitative difference in the toxicity against malignant and normal tissues.

5-Fluorouracil was developed by Heidelberger in 1957 following the observation that some tumours demonstrate enhanced incorporation of uracil into nucleic acids as compared with normal tissues (Rutman et al, 1954). It was hoped that this would provide a basis for the development of an antimetabolite with a selective toxicity against tumour cells (Heidelberger et al, 1957). However, 5-fluorouracil, like most other cancer chemotherapeutic agents, has a low therapeutic

Fig.1.1

Only one in five of those with cancer respond to a drug called Flouracic, while four out of three suffer severe side effects.

—*The Observer*

From Doctor Jekyll,

World Medicine, March 10 (1979), p.87.

index (Calabresi and Parks, 1975) i.e. the therapeutic dose is close to that which produces toxicity in the patient. Indeed, Moertel and Reitemeier (1967) state that 5-fluorouracil is only of therapeutic value at a dose greater than that which produces toxicity in non-malignant tissue.

Gastrointestinal toxicity is the first indication of the systemic toxicity of 5-fluorouracil. The symptoms include stomatitis, diarrhoea, anorexia, nausea and weight loss and these can be so severe that they limit the dose of 5-fluorouracil that can be used (Calabresi and Parks, 1975). Leukopenia usually succeeds the symptoms of gastrointestinal toxicity and also is a major cause of the termination of treatment (Calabresi and Parks, 1975). Since the low white cell count results in a reduced capacity of the immune system and hence an increased susceptibility to infection both of these side effects of the drug are highly undesirable. Especially so since cancer itself is associated with anorexia and malnutrition (Costa, 1977). The severity of the systemic toxicity of 5-fluorouracil has been noted (even if misprinted) in the popular press (Fig. 1.1)!

It is hard to give an exact figure for the success rate of treatment with 5-fluorouracil. Moertel and Reitemeier (1967, p. 930) show that, in 19 separate trials, claims of objective regression ranged from 8% to 85%. However, it is generally stated that tumour regression is achieved in only about 25% of patients selected for treatment with 5-fluorouracil (Haskell, 1980, p. 88; Heidelberger, 1973). This low response rate is due, in part, to the resistance of some tumours to 5-fluorouracil. 5-Fluorouracil itself is not toxic to cells and is only toxic after conversion to 5-fluoro-2-deoxyuridilate by a group of enzymes including thymidine kinase, uridine

kinase and phosphoribosyltransferase. Although these enzymes are present in normal mammalian cells they may be absent from tumour cells (or present in a less active form) and this might explain the resistance of some tumours to 5-fluorouracil (Myers et al, 1976). However, the low response rate is probably also due to the systemic toxicity of the drug which prevents its use at maximal therapeutic doses.

It is clear, therefore, that the potential value of 5-fluorouracil and of other chemotherapeutic agents could be increased if it were possible to reduce the toxic side effects of the drug without impairment of the therapeutic activity. This thesis describes such an approach to enhancing the therapeutic value of 5-fluorouracil. In this thesis the effects of 5-fluorouracil on the intestine have been studied in detail and attempts have been made specifically to alleviate the intestinal toxic side effects.

Gardner et al (1978) suggested that the malnutrition and diarrhoea associated with 5-fluorouracil administration might be due to impaired intestinal absorption. They showed that although the absorption of both glucose and water by the small intestine of the rat were transiently increased by 50% on the first day after administration of 5-fluorouracil they were decreased by 60% on the third day. There were parallel changes in intestinal peptide hydrolase activities and both enzyme and absorptive activities had recovered to above normal values 21 days after drug administration. Since Bounous et al (1971a) reported a marked decrease in the food intakes of rats after administration of 5-fluorouracil, Gardner et al (1978) suggested that the increase in absorptive and enzymic activities observed 24 hours after 5-fluorouracil administration

might be due to starvation, but that this was unlikely to be the cause of the reduction observed on the third day. However, the literature on the effects of starvation and food restriction on intestinal absorption is confused and contains reports of both increased and decreased absorption rates (e.g. Kershaw et al, 1960; Levin, 1970; Levin et al, 1965; Newey et al, 1970). Furthermore, from their results Gardner et al (1978) were unable to determine whether the impaired absorption which they observed was due to a change in the number of absorbing cells or a change in the absorptive capacity of individual absorbing cells. This is an important question and needs consideration. However, as discussed in Chapter 8, the resolution of this question is much more difficult than previous authors have implied.

Therefore, before attempts are made to reduce the intestinal toxicity of 5-fluorouracil, it is essential that the effects of 5-fluorouracil per se on the intestine are characterised in detail and that these are dissociated from the effects of the reduced food intake which follows administration of the drug. Hence, a part of this work has been concerned with the effects of starvation and semi-starvation on the intestine; in particular, attention has been paid to the choice of a reference basis for expression of absorption rates since it is apparent that this factor may be responsible for much of the seemingly conflicting information in the literature.

A major development arose in 1971 when Bounous and his associates in Sherbrooke, Canada, claimed that an 'elemental' diet could reduce the systemic toxicity of 5-fluorouracil. 'Elemental' diets are chemically defined diets composed of relatively simple nutrients which require little or no digestion prior to absorption. Bounous et

al (1971a and b) suggested that the impaired intestinal peptide hydrolase activities observed after 5-fluorouracil administration prevents digestion of proteins and that an 'elemental' diet is of benefit to the intestine because it supplies amino acids directly to the mucosal cells without the need for prior digestion. Since Hirshfield and Kern (1969) demonstrated that intestinal mucosal cells can incorporate amino acids supplied via the lumen to a greater extent than those supplied systemically, the suggestion of Bounous et al (1971a and b) seemed rational. Bounous and associates also claimed that 'elemental' diets were beneficial in protecting the intestine against ischaemic damage (Bounous et al, 1967) and against irradiation damage (Hugon and Bounous, 1972). These claims, if proven, would have enormous significance in several areas of medicine and so it is essential that they be evaluated thoroughly. Therefore Gardner and Heading (1979) studied the effects of 'elemental' diets on the intestinal toxicity of 5-fluorouracil. Although they failed to demonstrate a beneficial effect of 'elemental' diets there were a number of inconsistencies between their study and that of Bounous et al (1971a). In view of the benefits to be gained by reduction of the systemic toxicity of 5-fluorouracil this approach clearly merited further investigation. Furthermore, although 'elemental' diets are used in the treatment of a number of disorders of the gastrointestinal tract (Koretz and Meyer, 1980) little is known about the effects of 'elemental' diets on the physiology of the small intestine, and it is clearly desirable to study these in detail. The aspects which have received particular attention in this work include the effects on mucosal peptide hydrolase activities, the relationship between body weight and food intake for various diets, the possible affect of dietary flavouring on an ad libitum regime, and the feeding pattern

(i.e. the hour-to-hour consumption of food).

It has already been claimed that the therapeutic efficiency of chemotherapeutic agents can depend on the dose schedule used (e.g. Ansfield and Greenspan, 1979) although this is not often exploited in clinical practice. Chemotherapeutic agents can be divided into two groups depending on whether they are toxic only to cells in a specific phase of the cell division cycle, e.g. cytosine arabinoside and methotrexate which are specific for cells in S-phase, or whether they are toxic in all phases of the cell division cycle but may be more toxic to proliferating cells than to non-proliferating cells, e.g. 5-fluorouracil (Heidelberger, 1973). Since most of these agents are rapidly degraded by the liver (Haskell, 1980) it is clear that those specific for a single phase of the cell division cycle will be most effective when administered at a time when most of the tumour cells are in the necessary phase of the cell cycle. This rationale has been used as the basis for the development of dose schedules for a number of chemotherapeutic agents (van Putten, 1974). Furthermore, Skipper et al (1967) reported that the ability of cytosine arabinoside to kill mouse leukaemia cells could be greatly increased if the timing of sequential doses of the drug was such that they coincided with the S-phase of the cells division cycle.

Although this approach has been applied in attempts to improve the therapeutic efficiency of these agents, little attention has been paid to the possibility that a similar approach could be used to reduce the toxicity to normal tissues of chemotherapeutic agents. A major part of this work has, therefore, been devoted to such an approach to minimizing the intestinal toxicity of 5-fluorouracil and to answering the question "Can the intestinal toxicity of

5-fluorouracil be reduced by selection of a particular time of drug administration?" Positive results will be presented which give grounds for cautious optimism that the therapeutic value of drugs such as 5-fluorouracil may indeed be increased, perhaps dramatically, by means as simple as the selection of an optimum time for drug administration.

Aims of the study

The principle aims of this study can be summarised thus:

- (1) To establish the pathophysiology of the effects of 5-fluorouracil on the small intestine and to dissociate the effects of 5-fluorouracil per se from those of the consequent reduced food intake.
- (2) To determine the effects of 'elemental' diets on the physiology of the small intestine and to determine whether an 'elemental' diet is of benefit to the intestine during 5-fluorouracil administration.
- (3) To investigate alternative methods to reduce the intestinal toxicity of 5-fluorouracil; in particular the possibility that alteration of the animals feeding time or of the time of administration of the drug might be beneficial.

Lay-out of thesis

Each chapter begins with a self-contained Introduction and therefore details of the rationale behind the various stages of the work will not be discussed here.

Gardner et al (1978) had already established a suitable animal model for the study of the effects of 5-fluorouracil on the small

intestine. This model seemed to be well suited to the requirements of the present study and therefore it has been adopted throughout this work. A high dose of 5-fluorouracil is used and this induces substantial intestinal toxicity.

Gardner et al (1978) and Gardner and Heading (1979) estimated intestinal absorption of glucose and water as indices of the functional capacity of the small intestine. Furthermore, they showed that absorption rates of glucose and water were highly correlated. Since intestinal water absorption rates can be estimated rapidly and reproducibly by the technique of Fisher and Gardner (1974), water absorption is used throughout this work as the principle index of the functional capacity of the small intestine and the method is described in Chapter 3.

The rationale behind the claim by Bounous et al (1971a and b) that 'elemental' diets are of benefit to the intestine during 5-fluorouracil administration was based on the observation that intestinal peptide hydrolase activities are decreased after 5-fluorouracil administration. A method was therefore required for the estimation of these activities. Several methods have been described in the literature. These have been compared and various modifications tested. It became clear that the thermo-lability of the peptidases creates special problems in their estimation which can be minimized by adoption of a rigorously standardised technique. Also, preliminary trials showed that a highly sensitive assay was required if peptide hydrolase activities were to be measured in luminal effluents from perfused intestines. Chapter 4 therefore describes the development of a satisfactory assay for peptide hydro-

lase activities.

Levin (1968) suggested that an important effect of 5-fluorouracil was a reduction in the number of cells in the intestine, and this too was implicit in the histological appearance of intestines from 5-fluorouracil-treated rats mentioned by Gardner et al (1978). In an attempt to determine whether the impairment of absorptive activity is due to a decrease in the number of absorbing cells the value of estimating cell numbers by measuring the mucosal DNA content, as suggested by Batt and Peters (1976), is considered. Minor modifications were made to improve an established method for DNA estimation and it became clear that simple precautions were necessary if DNA analyses were to be reliable. Chapter 5 describes the method used for the estimation of DNA.

Chapter 6 describes the effects of luminal perfusion on the intestinal mucosal DNA content and peptide hydrolase activities. This study was designed primarily to test whether a perfused intestine could be used for the estimation of mucosal DNA content and peptide hydrolase activities. However, it also gives useful information about the physiological significance of the release of peptide hydrolase activities from the small intestine, a matter of considerable importance to those workers studying peptide transport or the effects of peptide hormones on the intestine. Since the results suggested that intestinal hypoxia might promote the release of peptidases from the mucosal cells, a short investigation (Chapter 7) was carried out to examine whether this might be the basis of a possible novel method for the clinical diagnosis of intestinal ischaemia.

The effects of 5-fluorouracil and of starvation on the small intestine are described in Chapter 8. Also discussed in this chapter are the effects of the use of different reference systems for the expression of absorption rates and the possibility that this may explain, in part, the confused literature on the effects of starvation on the small intestine.

During the course of this work a different salt form of 5-fluorouracil became available and an unexpected finding was that the two salt forms of 5-fluorouracil have markedly different systemic toxicities. Chapter 9 describes experiments which show that the plasma pharmacokinetics of the two salts were different, an interesting but inexplicable finding.

Chapter 10 describes the effects of 'elemental' diets on the physiology of the small intestine. The possibility that these diets might reduce the intestinal toxicity of 5-fluorouracil is considered in detail, but it is concluded that, on balance, there are more likely to be adverse effects of 'elemental' diets than benefits.

Chapter 11 describes an attempt to reduce the intestinal toxicity of 5-fluorouracil by alteration of either the time of drug administration or the time of feeding. This approach has been successful and leads to the suggestion that a similar approach in humans might lead to a significant improvement in clinical chemotherapy.

The final conclusions of this work are presented in Chapter 12.

CHAPTER 2

General Methods: Animals, diets and 5-fluorouracil administration

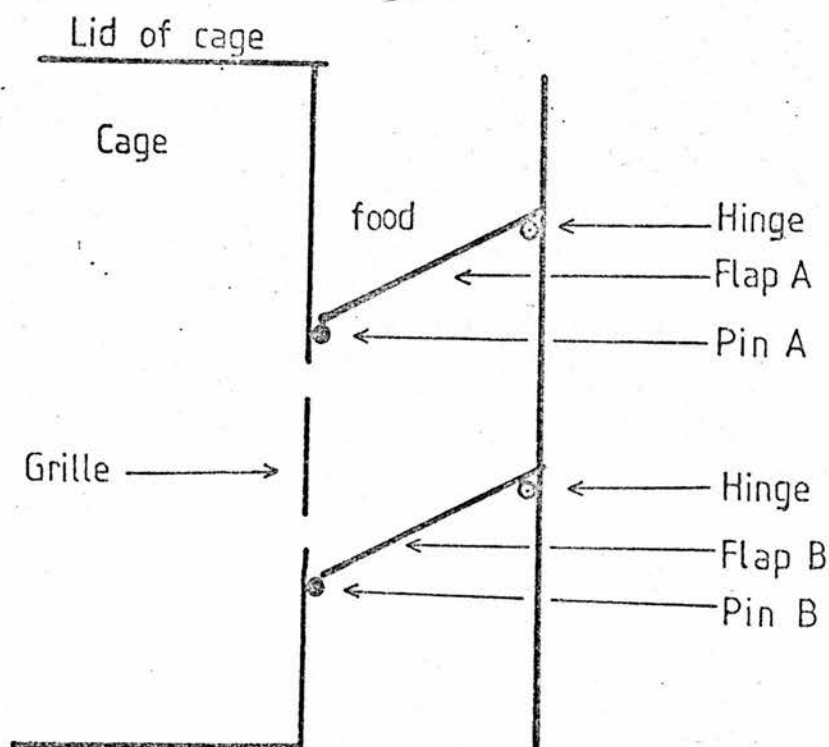
Animals

Throughout this work male and female rats of a local Wistar strain, bred at the Centre for Laboratory Animals, Easter Bush, Penicuik, Midlothian, were used. The animals, weighing between 140 and 160 g, were kept in the Animal House of the Department of Biochemistry under conditions of controlled temperature and day length with a light period of 12 hours from 07:00 to 19:00. Free access to water was allowed at all times and for the first 7 days and thereafter unless otherwise stated, all rats had free access to a standard pelleted diet, Oxoid Diet 86, (Oxoid Ltd., Basingstoke, Hants), hereafter referred to as 'Oxoid diet' for brevity. Coprophagy was not prevented.

Animals were kept for at least 7 days before they were used for experiment in order to minimize possible variability due to rehousing of the rats.

Caging of animals

When free access to the Oxoid diet was allowed throughout the experiment up to 8 rats were caged together. However, in some experiments in which access to food was limited to a short period each day, or in which rats were fed on an 'elemental' diet, a maximum of 4 rats were caged together. When 5 or more rats were caged together at least one rat failed to gain weight or gained much less weight than normal (see Chapter 11 for details).



Automatic feeding device (elevation)

Fig. 2.1 Automatic feeding device used to restrict access to food to only a single 3 hour period each day. The device is pre-set each day in the position shown. At the start of the feeding period Pin A is withdrawn by the action of a solenoid and the rats have access to food through the grille. At the end of the feeding period Pin B is withdrawn by a second solenoid and the food drops to a container below such that the rats no longer have access to food. The solenoids are actuated by a Sangamo Weston time-switch. (The apparatus was designed by Professor R.B. Fisher and Dr. M.L.G. Gardner for use in a previous study, Fisher and Gardner, 1976)

Diets

In some experiments the standard diet was replaced by an 'elemental' diet. The diets used were either the two commercially available diets 'Vivonex-HN' (Eaton Laboratories) and 'Flexical' (Mead-Johnson) or a 'home-made' diet, Diet B, as used by Bounous et al (1971a). The compositions of these diets are given in Chapter 10. Animals had free access to the diet unless otherwise stated and the 'elemental' diets were presented in dry form in a glass bowl in the cage.

Automatic feeder devices

In some experiments in which access to food was limited to 3 hours each day a pair of automatic feeding devices were used. One of them had been used previously by Fisher and Gardner (1976) for a similar purpose and they were built by the work shops of the Department of Biochemistry.

The device is shown in diagrammatical form in Fig. 2.1. Two pins, A and B, hold in position flaps A and B respectively. The pins are connected to solenoids which are operated by a time switch. The cage is pre-set in the position shown in Fig. 2.1. At the start of the feeding period Pin A is withdrawn by operation of the solenoid and Flap A drops to release the food as far as Flap B such that the rats have access to food through the grille. At the end of the feeding period Pin B is withdrawn by a second solenoid and the food drops to a container below such that the rats no longer have access to food.

Only 4 rats could be housed in each cage (see above) and the

device was suitable for use only with the pelleted Oxoid diet.

Choice of sex of rats

The sex of rats used is stated in all experiments. Fisher (1955) reported that water absorption rates by the isolated perfused small intestine from female rats were significantly greater, by 25%, than those from male rats. As a result of this observation female rats have been used routinely in this laboratory for the study of intestinal absorption. However, results are presented in Chapter 8 which demonstrate that there is no significant difference between absorption rates estimated on intestines from male and female rats perfused in vitro by the method of Fisher and Gardner (1974). Also, Crocker (1971) demonstrated that water absorption rates estimated on intestines from female rats vary during the four day oestrus cycle. Although in retrospect no difference was detected in the variability of absorption rates in intestines from male and female rats during the present study, the effects of 5-fluorouracil on the oestrus cycle are not known. Therefore, in order to avoid possible variation from this source male rats have been used in most of the experiments which involve 5-fluorouracil administration. Furthermore, the growth rates and food intakes of male rats are greater than those of female rats (see Chapter 10) and therefore changes are easier to measure in males. An added advantage of male rats is that they are more docile during handling.

5-Fluorouracil administration

Light ether anaesthesia was induced by placing the pre-weighed rat in a glass dessicator which had a pad of ether soaked cotton wool in the bottom, covered by a disc of wire gauze. The rat was

removed and injected intraperitoneally with a sterile solution of 5-fluorouracil (Tris or sodium salt, supplied by Roche Products Ltd, Welwyn Garden City, as sterile aqueous ampules for clinical use) at a mean dose of 1.44 mmol/kg body weight unless otherwise stated. All rats were placed in clean cages after injection and daily food intakes and body weights were recorded at 10:00.

A standard injection time of 10:00 was chosen for convenience; in some experiments the injection time was varied (Chapter 11) and all rats were injected within 10 minutes of the specified injection time.

All times refer to Greenwich Mean Time and precautions were taken to ensure that the control clocks for the lighting were not altered to British Summer Time.

Statistical analysis

The significance of the differences between mean values has been tested by the unpaired Student's t-test unless otherwise stated. Before use of the test care was taken to confirm that the data were normally distributed, as far as could be detected by visual inspection. Formal tests for deviation from normality (e.g. kurtosis, skewness - see Snedecor and Cochran, 1959, p. 199) were not applied since large numbers of data would have been required. Nevertheless, it can be noted that the t-test (unlike variance ratio tests) is relatively robust towards deviations from normality (Kendall and Stuart, 1973, p. 483).

CHAPTER 3

Estimation of water absorption by isolated rat small intestine

Introduction

In this work, intestinal absorption is being used as an index of the functional capacity of the small intestine and a method for accurate measurement of absorption rates was required. There are a number of methods available for the study of intestinal absorption both in vivo and in vitro and these have been reviewed by Parsons (1968). However, none of the techniques are ideal and therefore the choice of method depends upon the requirements of the proposed study.

The study of intestinal absorption in vivo is complicated by factors unrelated to the actual transfer capacity of the mucosa such as gastric emptying, intestinal motility, intestinal blood flow and hormonal action. While these factors are relevant to studies of the control of intestinal absorption in vivo they can result in uncontrolled variability in studies of absorption in vivo. Furthermore, most techniques in vivo measure absorption as a net loss of solute or fluid from the intestinal lumen and equate this loss with transfer from the intestinal lumen to the blood vessels and lymph on the serosal surface of the intestine. This is not always justifiable since some substances are accumulated in the mucosal cells during transfer from the intestinal lumen to the blood, e.g. iron and vitamin B₁₂ (Callender, 1967; Matthews, 1967), and others are metabolized before entering the blood as in the case of disaccharides, dipeptides and glycerides, all of which are hydrolysed by the mucosal cells (see Levin and Smyth, 1963). A great advantage of preparations

in vitro is that the environment of the intestine can be carefully controlled and, in some, there is access to both mucosal and serosal fluids.

One of the major problems in the development of intestinal preparations in vitro was that of supplying sufficient oxygen to the mucosal cells since the intestinal mucosa has a very high oxygen requirement (Wilson and Wiseman, 1954a). Fisher and Parsons (1949) developed one of the first successful intestinal preparations in vitro by providing a continuous supply of oxygen to the intestinal mucosa from an oxygenated Krebs bicarbonate solution circulated through the lumen of the intestine and from another circulated around the serosal surface. They believed that the success of their preparation was due partly to the fact that luminal perfusion was established in the anaesthetized rat prior to removal of the intestine so that it was never deprived of a supply of oxygen. This precaution cannot be adhered to with some other intestinal preparations in vitro such as everted sacs (Reid, 1901; Wilson and Wiseman, 1954), tissue rings (Agar et al., 1956) and tissue slices (Kimberg^{et al.}, 1961).

Both Fisher and Parsons (1949) and Fisher and Gardner (1974) advocated the use of precautions to prevent temporary hypoxia during studies of intestinal absorption in vitro. Fisher and Gardner (1974) showed that a delay of 15 minutes between excision of the intestine and establishment of luminal perfusion was detrimental to subsequent absorption of glucose and water although the damage was slowly reversible. Furthermore, Gardner (1978) demonstrated a 50% reduction in both glucose and water absorption rates when intestines for perfusion were removed from freshly killed rats instead of from ether-anaesthetized rats after establishment of luminal perfusion with a

segmented flow of perfusate and bubbles of oxygen. These observations demonstrate clearly the value of the precautions to avoid temporary hypoxia and suggest that absorption might be impaired and experimental variability increased in preparations in vitro such as everted sacs and tissue rings where these precautions cannot be applied.

During luminal perfusion the intestine should be maintained at a distension pressure of about 30 cm water in order to ensure adequate exposure of the mucosal surface of the intestine to the oxygenated medium. Distension is achieved in everted sacs by the pressure of the fluid within the sacs and occurs naturally in intestinal rings cut from everted intestine due to contraction of the smooth muscle in the intestinal wall (see Wilson, 1962, p. 35). However, Fisher and Gardner (1974) showed that maximal absorptive activity could not be maintained in isolated small intestine perfused with a continuous flow of oxygenated perfusate and they suggested that this effect was due to the slow rate of diffusion of oxygen in the luminal perfusate. This limitation also applies to other preparations in vitro such as everted sacs, tissue rings and tissue slices. Furthermore, Fisher and Gardner (1974) showed that, when the flow of oxygenated perfusate was broken by bubbles of oxygen, absorption no longer appeared to be limited by luminal diffusion. This so-called 'segmented-flow' not only provides additional oxygen to the mucosal cells in the form of bubbles of 5% carbon dioxide in oxygen but also results in a rapid mixing of the luminal perfusate during perfusion. Winne (1979) subsequently confirmed that the segmented-flow, used by Fisher and Gardner (1974), causes a marked reduction in the calculated thickness of the unstirred layer adjacent to the mucosal cells which occurs either

with the laminar flow characteristics of a continuous flow of perfusate or with other in vitro incubation techniques (Wilson and Dietschy, 1974). This unstirred layer results in slow diffusion between the medium and the mucosal cells and hence impairment of absorption rates, and is a major limitation inherent in many techniques for the study of absorption. The implication of an 'unstirred layer' is that the concentrations of solutes in contact with the absorbing surface of the cells are not the same as those in the bulk fluid within the lumen. Further, as noted above, the diffusion across this 'unstirred layer' (and not the transport process) can become rate-limiting for intestinal absorption. A further advantage of the segmented-flow of luminal perfusate is that it allows a sufficiently low perfusion rate so that the concentration difference between the perfusate and luminal effluent allows the precise measurement of absorption (Fisher and Gardner, 1974).

While Fisher and Gardner (1974) showed that their preparation continued to concentrate glucose for more than 5 hours, Levine et al (1970) demonstrated morphological destruction of everted sacs after incubation for only 5 minutes. Also, there is evidence that eversion of the intestine increases its permeability to some solutes (Chalfin et al, 1958) and to inulin and phenol red which are practically impermeable in vivo (Smyth and Taylor, 1957) and decreases the potential difference across the rat jejunum (Baker et al, 1969). Furthermore, Silk and Kim (1976) suggested that studies of peptide absorption in vitro may not be valid due to an unphysiological release of peptide hydrolase activities from these preparations. As discussed in Chapter 6, this release might be the

result of temporary hypoxia since the release is much less from intestines perfused in vitro by the method of Fisher and Gardner (1974) provided that their precautions to avoid temporary hypoxia are observed.

However, experiments with preparations in vitro such as everted sacs, tissue rings and tissue slices have made a significant contribution to the understanding of the mechanisms of intestinal absorption. These preparations have the advantage of simplicity since the apparatus requirements are minimal. Furthermore, a large number of experiments can be carried out on a single intestine thereby reducing inter-animal variability. In contrast, a large segment of intestine is required for perfusion in vitro in order to obtain sufficient secretion from the serosal surface and to obtain a large concentration difference between the perfusate and luminal effluent. Since it is difficult to measure a constant length of the small intestine in situ the whole jejunum plus ileum are routinely used in this laboratory; this also avoids the problem of variability of the absorptive capacity of different regions of the small intestine. It is, however, possible to carry out more than one experiment on each intestine by the method of Fisher and Gardner (1974). Segmentation of the luminal perfusate allows the perfusate composition to be changed with a corresponding rapid change in the composition of the luminal effluent so that a new steady state is established within about 5 minutes (Fisher and Gardner, 1974). This permits the use of at least two perfusate compositions with each intestine, although this has not been exploited in the present work.

One problem associated with all intestinal preparations in vitro is that of accumulation of solutes in the tissue fluid to unphysio-

logical levels due to the absence of the serosal blood flow. Fisher and Gardner (1974) showed that tissue accumulation of a solute was dependent upon the rate of water absorption and that it was only when absorption rates were high (greater than 150 $\mu\text{l}/\text{cm}/\text{hr}$ for the proximal 40 cm of the jejunum plus ileum) that the cumulative appearance of a solute in the serosal fluid was a reliable measure of absorptive activity. They found that when the glucose concentration in the luminal perfusate was 28 mmol/l the tissue fluid concentration rose to about 70 mmol/l. Furthermore, Fisher and Gardner (1974a) demonstrated that the rate of glucose absorption was increased when the tissue accumulation was reduced by the introduction of an arterial infusion technique, and they suggested that maintenance of physiological tissue fluid glucose concentration may be important for the functioning of the small intestine. This problem is partly overcome in this and other preparations in vitro by the use of short experimental periods, although this may introduce further errors since Fisher and Gardner (1974) demonstrated that their preparation requires a short period of about 5 minutes to become stable.

The technique of Fisher and Gardner (1974) was therefore chosen as the most suitable method for the measurement of intestinal absorption *in vitro*. Intestines are perfused by a segmented-flow of bubbles of 5% carbon dioxide in oxygen and slugs of oxygenated perfusate in a single-pass through the lumen. The mesentery is removed so that the fluid and solutes which would enter the portal circulation and the lymphatics can be collected directly. Intestines are removed from ether-anaesthetized rats only after establishment of luminal perfusion such that the intestine is never deprived of a supply of oxygen. This preparation is stable for at least 100 minutes (Fisher

and Gardner, 1974). Furthermore, the apparatus for this technique was already available and there existed in the laboratory considerable knowledge of the properties of the preparation and expertise in its use.

Since the appearance of a solute in the serosal fluid is dependent on the water absorption rate and since Gardner et al (1978) and Gardner and Heading (1979) demonstrated a highly significant correlation between the rates of glucose and water absorption, water absorption rates have been used throughout this work as an index of the functional capacity of the small intestine. A further advantage of the use of water absorption rates is that these are estimated directly from the volume of fluid secreted on to the serosal surface of the intestine. Estimation of the rate of absorption of glucose or other solutes requires a further analysis of the contents of the luminal effluent and thus an additional error in estimations of absorption rates. It must be stressed that estimation of water absorption rates with most of the alternative preparations in vitro requires the use of an 'unabsorbable' marker substance such as polyethylene glycol which may be adsorbed on to the mucosal cells and may even decrease absorption rates (Davis et al, 1980).

This chapter describes the estimation of water absorption rates by the method of Fisher and Gardner (1974).

Estimation of water absorption rates

Throughout this work water absorption rates were estimated on the whole jejunum plus ileum from either male or female rats. Since there is a diurnal rhythm in the absorption of glucose and water by the rat small intestine (Fisher and Gardner, 1976) all absorption experiments were performed between 10:00 and 16:00.

Perfusate

This was a Krebs and Henseleit (1932) bicarbonate saline solution modified by halving the concentration of calcium and quartering that of magnesium. The following stock solutions were prepared with AnalaR chemicals (B.D.H. Chemicals Ltd) and stored at 4°C:

Stock perfusate	69.25 g NaCl, 20.90 g NaHCO ₃ , 3.53 g KCl, 1.61 g KH ₂ PO ₄ , 0.73 g MgSO ₄ ·7H ₂ O in 2 litres of doubly distilled water.
CaCl ₂	13.9 g CaCl ₂ ·6H ₂ O in 500 ml doubly distilled water.
Phenol red	1.0 g phenol red dissolved in 1 litre of doubly distilled water with 1 pellet of NaOH added and then filtered.
NaCl	140.2 g NaCl in 1 litre of doubly distilled water.
NaHCO ₃	42.0 g NaHCO ₃ in 1 litre of doubly distilled water.

The perfusate was prepared freshly from 200 ml of stock perfusate, 50 ml phenol red and 5 g glucose. The volume was made up to about 950 ml with doubly distilled water and 10 ml of the stock CaCl₂ solution was added and the volume made up to 1 litre. The final concentration of glucose and phenol red was 28 mmol/l and 141 nmol/l

respectively and the pH of the perfusate once equilibrated with 5% carbon dioxide in oxygen was 7.4. Phenol red was included in the perfusate to indicate the presence of leaks in the intestine.

The lumen of the intestine was rinsed with a bicarbonate saline solution (NaCl 120 mmol/l : NaHCO_3 25 mmol/l) prepared from 50 ml of each of the stock solution made up to a volume of 1 litre with doubly distilled water and equilibrated with 5% carbon dioxide in oxygen.

Anaesthesia

Anaesthesia was induced by placing the rat in a glass dessicator which contained a pad of ether soaked cotton wool covered by a disc of wire gauze. The animal was removed from the dessicator and placed supine on the operating table on a pad of cellulose wadding B.P.C. ('Cellozene' supplied by Robinsons of Chesterfield). Its feet were tied down and its nose and mouth placed in an open ended glass tube which contained a pad of ether soaked cotton wool. The pad of cotton wool was removed at intervals so that the animal was maintained under light ether anaesthesia while the intestine was connected to the perfusion apparatus. Only when perfusion was established was the intestine removed from the rat and the animal killed. If the animal died prior to establishment of perfusion the experiment was abandoned.

Setting up the intestine

The abdomen was opened by a midline incision and a black polyethylene drape laid over the rat. The intestine was exteriorised through a central slit in the drape and the oral cannula was tied into the upper end of the jejunum; this was located as the highest part of the jejunum which could be withdrawn from the abdominal

cavity without traction on the ligament of Treitz. Warm oxygenated saline was used to wash out the luminal contents and a second cannula was tied into the distal end of the ileum just proximal to the ileo-caecal valve.

The perfusion apparatus

The apparatus is shown diagrammatically in Fig. 3.1.

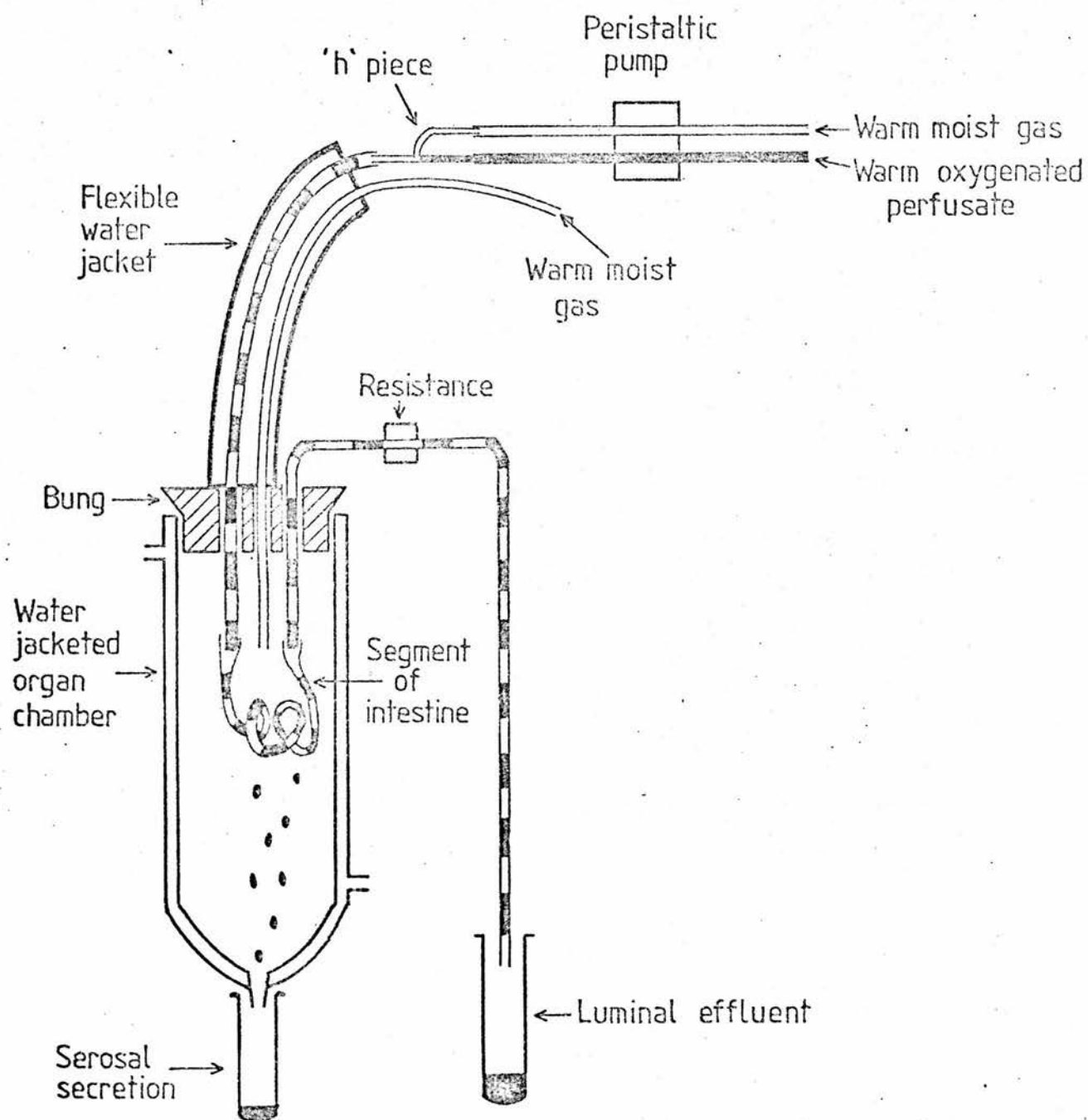
The perfusate and bicarbonate saline are contained in two glass reservoirs, either water jacketed or immersed in a water bath at 41°C. They are equilibrated with 5% carbon dioxide in oxygen by means of gas lifts which carry approximately 100 ml gas per minute into each reservoir. The perfusate and saline are warmed and equilibrated with the gas mixture for at least 1 hour prior to use.

Warm moist gas (5% carbon dioxide in oxygen) is pumped from the saline reservoir by a peristaltic pump (Watson-Marlow Delta multi-channel) into a stream of warm oxygenated perfusate delivered by a second channel on the pump to form a sequence of slugs of liquid separated by gas bubbles, the so-called segmented-flow. The pumping rate was normally 3.5 ml/minute for each of perfusate and gas.

Warm oxygenated saline for washing out the segment of intestine is pumped by an independent peristaltic pump ('Pericyclic' pump by Schuco Scientific Ltd, London). A by-pass line with a relief valve (a modified Starling resistance, Fisher and O'Brien, 1972) allows saline to return to the saline reservoir if the luminal distension pressure exceeds 30 cm water.

The segmented-flow of perfusate passes through a flexible water jacket (37°C) connected to the bung of the organ chamber. The bung is

Fig. 3.1 Perfusion apparatus. A flow of warm moist gas (5% carbon dioxide in oxygen) is pumped by a peristaltic pump into a stream of oxygenated perfusate via an 'h' piece (Technicon Instruments Co., Chertsey, Surrey) to form a sequence of slugs of perfusate and bubbles of gas (the 'segmented-flow'). The segmented-flow of perfusate passes through a flexible water jacket and is delivered to the inlet cannula tied into the top end of the jejunum. The outflow from the segment of intestine passes through a resistance which maintains the intestine at a distension pressure of 30 cm water and is collected into tared tubes. The intestine is suspended in a water jacketed organ chamber maintained at 37°C. A stream of warm moist gas (5% carbon dioxide in oxygen) passes via the flexible water jacket through the organ chamber. This gas supplies oxygen to the luminal surface of the intestine and blows the secretion from the serosal surface of the intestine along the exit tube at the bottom of the organ chamber to tared collecting tubes.



removed from the organ chamber and placed on the operating table beside the rat and the segmented flow is delivered to an inlet cannula of glass tied into the jejunum. The ileal cannula is connected to the outlet tube which passes through the bung and the pump speed is increased three fold until luminal perfusion is established.

A modified Starling resistance is connected to the outflow tubing to maintain a distension pressure of 30 cm of water so as to expose uniformly the whole mucosal surface to the perfusate and gas.

Luminal perfusion is established once the segmented-flow passes through the resistance and the pump rate is then reduced to about 3.5 ml/minute and the intestine is removed from the anaesthetized rat. The mesentery is clamped adjacent to the caecum and divided peripherally to the clamp and the remnant is gently torn away from the segment of small intestine. The intestine is dipped into a bowl of warm oxygenated saline to remove traces of blood from the serosal surface and a length of nylon yarn is pulled through the naturally formed loops of intestine. The organ chamber bung is then replaced and the intestine is suspended, free from kinks, in the water jacketed organ chamber. This procedure is normally completed within 5 minutes of induction of anaesthesia.

Gas (5% carbon dioxide in oxygen) saturated with water vapour at 37°C is carried through the flexible water jacket and passes through the organ chamber. This gas is passed through two traps, one just before the water jacket and one at the outlet inside the organ chamber, to prevent condensed water vapour passing into the serosal secretion. The flow of gas serves to oxygenate the serosal surface

of the intestine and to blow the transported fluid (the serosal secretion) along the exit tube at the bottom of the organ chamber to tared collecting tubes. The outflow from the intestinal lumen (the luminal effluent) passes to a second set of tared collecting tubes. The two sets of tubes are held in a time operated fraction collector (Central Fraction Collector, supplied by Gallenkamp and Co. Ltd) modified as described by Gardner (1971).

In the original perfusion unit of Fisher and Gardner (1974) the perfusate and saline reservoirs are immersed in a water bath at 41°C and this water is circulated through the water jackets by a pump ('Circotherm' supplied by Shandon Southern Ltd, Camberley, Surrey). An alternative design employs a flow heater (Model FH15 Grant Instruments Cambridge Ltd) to circulate warm water through the water jackets and in this design the reservoirs are also water jacketed.

Estimation of intestinal length and dry weight

After completion of perfusion the intestine was removed from the perfusion apparatus. The length was estimated with the intestine laid along the length of a metre rule and held straight between the cannulae but not under tension. The cannulae were removed and the intestine placed on a piece of tared aluminium foil and dried to constant weight at 75°C in an oven.

Absorption rates

Because the water content of the intestine remains constant throughout the experiment (Fisher and Gardner, 1974) it is assumed that the total volume of liquid pumped into the lumen during any collection period is equal to the sum of the volume of effluent

issuing from the lumen plus the volume of secretion. Water absorption rates are therefore calculated directly from the volume (ie. weight) of secretion on to the serosal surface of the intestine in a given time.

The serosal secretion was collected over sequential 5 minute periods commencing immediately after setting up the intestine. Absorption rates became stable within 5 minutes and remained so over the next hour (Fig. 3.2). These rates agree closely with the values observed by Fisher and Gardner (1976) under the same conditions.

Normally four 5 minute collections were made of the serosal secretion from each intestine and the absorption rate was calculated from the last three collections.

Absorption rates are normally expressed in terms of $\mu\text{l}/\text{cm}/\text{hr}$. However, in Chapter 8, they are also expressed with reference to intestinal dry weight, mucosal DNA content and whole small intestine.

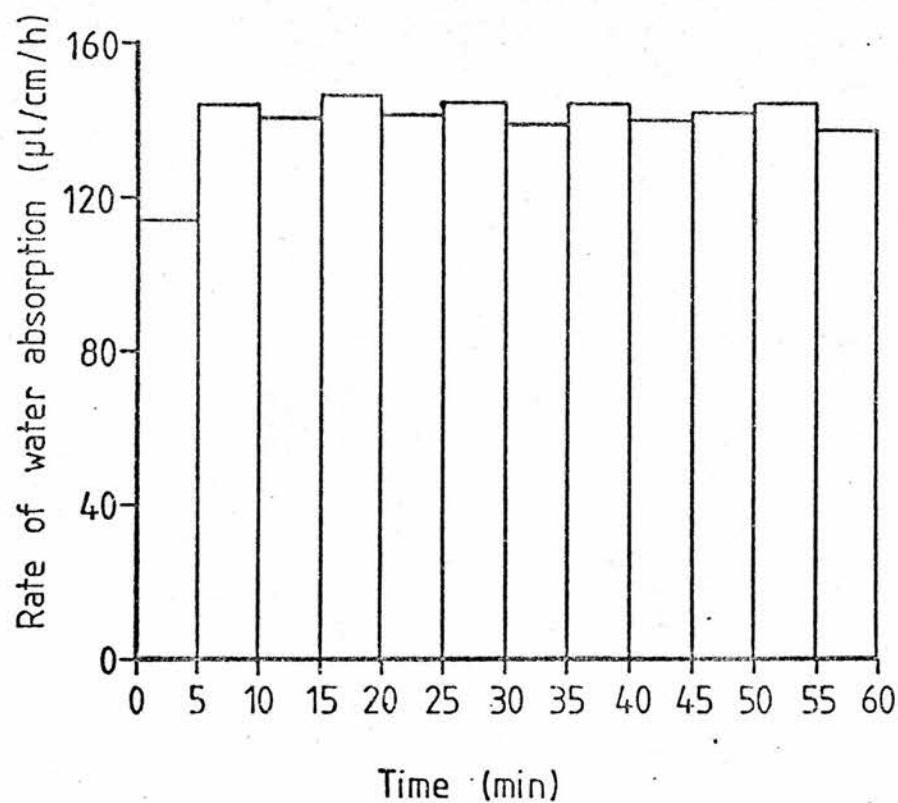


Fig. 3.2 Stability of the preparation. Rates of water absorption from the lumen were measured over successive 5 minute periods (mean of 4 experiments)

CHAPTER 4

Estimation of peptide hydrolase activities

Introduction

As described in the General Introduction it was desired to measure peptide hydrolase activities in the mucosal cytoplasm since impairment of these activities had already been shown by Bounous et al (1971a), Gardner et al (1978) and Gardner and Heading (1979); furthermore, this observation was central to the rationale behind the suggestion by Bounous et al (1971a and b) that 'elemental' diets were beneficial to the intestine during 5-fluorouracil administration.

An existing method was investigated and modified for use in these studies. Gardner et al (1978) and Gardner and Heading (1979) used a method modified from that of Fujita et al (1972) for the estimation of cytoplasmic peptide hydrolase activities in the intestinal mucosa. Their method involves two steps: in the first the material to be assayed is incubated in the presence of a dipeptide substrate and then, after termination of this reaction, the released amino acids undergo oxidative deamination by the action of L-amino acid oxidase from snake venom and the hydrogen peroxide so produced oxidises the chromogen, O-dianisidine, in the presence of peroxidase.

However, Gardner et al (1978) noted high variability in the activities estimated and they stressed the technical difficulties involved. These were mainly due to (a) loss or leakage of enzymes from tissues in vitro, (Josefsson and Sjöström, 1966; Lindberg et al, 1975; Silk and Kim, 1976) and (b) loss of activity during

handling of the tissue at room temperature since many peptidases are thermolabile. Furthermore, in order to be able to estimate absorptive and enzymic activities on the same intestine, Gardner et al (1978) used only 1 cm segments of proximal jejunum and distal ileum for the estimation of enzymic activities and expressed the activities found relative to protein.

Since peptide hydrolase activities are known to vary in different regions of the small intestine (Lindberg et al, 1975, p. 226; Robinson and Shaw, 1960), activities estimated on 1 cm segments might not be representative of the activity of the whole small intestine. Also, the use of short segments of intestine necessitates the use of a reference system such as unit length of intestine, unit weight of intestine or protein, all of which might be affected by the treatment under study thereby influencing the changes observed in enzyme activities. In order to avoid the use of such reference systems in this work enzyme activities are expressed as the total activity of the whole jejunum plus ileum. The advantages of this, and the consequences of the use of different reference systems, are discussed elsewhere in the specific context of the effects of starvation on absorption by the rat small intestine (Chapter 8).

The method used by Gardner et al (1978) was therefore modified for application to the whole jejunum plus ileum. Furthermore, strict control of temperature during preparation of the mucosal homogenate and preparation of the assay has been introduced in order to minimize thermal inactivation of the enzymes.

This method was used in the preliminary study, described in

Chapter 6, of the release of peptide hydrolase activities from the small intestine during perfusion in vitro and in vivo. The study had two purposes: (1) to test the application of the peptide hydrolase assay to the estimation of peptide hydrolase activities in the intestinal mucosa and (2) to determine whether an intestine which had already been perfused for measurement of absorptive activity could be used for the estimations. If the latter were possible, it would enable the estimation of water absorption and of peptide hydrolase activities on the same intestine and the advantages of this are discussed in Chapter 6. The method was found to have a number of shortcomings (see below) and therefore other published methods were explored.

The method of Fujita et al (1972) involves two consecutive incubations of 30 minutes and 60 minutes, for the peptide hydrolysis and the amino acid oxidase reactions respectively. Nicholson and Kim (1975) reported ^{one of which} methods, _h combines the two steps into a single incubation of 20 minutes. This method was investigated and compared with a similar one in which two 20 minute incubations are used (Nicholson and Kim, 1975). The latter method gave consistently higher activities than the former and it was concluded that the L-amino acid oxidase inhibits the peptide hydrolase activities estimated. Therefore, since the two step assay, described by Nicholson and Kim (1975), had several advantages over that of Gardner et al (1978) in terms of speed and sensitivity, it was chosen with minor modifications for use in this work.

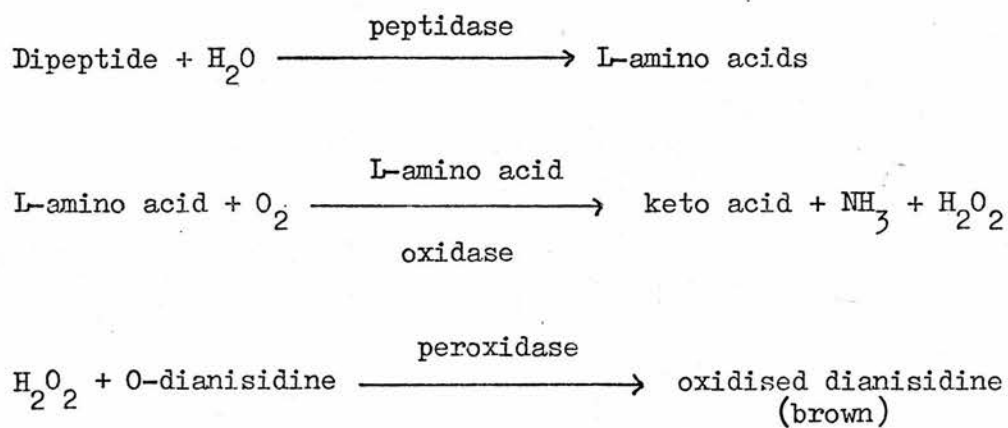


Fig. 4.1 The sequence of reactions involved in the estimation of peptide hydrolase activities.

Method '1': The method of Fujita et al (1972) as modified by Gardner et al (1978)

Principle of the method

Samples containing peptide hydrolase activity are incubated with dipeptide substrates. The liberated L-amino acids are oxidised by ophidian (snake) L-amino acid oxidase to the corresponding imino acids which spontaneously decompose to the keto acids, releasing ammonia. The reduced flavin moiety of the oxidase enzyme is reoxidised by molecular oxygen, and the resulting hydrogen peroxide is coupled by peroxidase to the oxidation of colourless O-dianisidine to a brown dye (see Fig. 4.1).

Snake venom L-amino acid oxidase does not oxidise dipeptides or larger peptides and only acts on some L-amino acids. Of the dipeptides chosen as substrates for the assay in this work, Gly-L-Met, L-Leu-Gly and L-Val-L-Leu, only one of each pair of amino acids (leucine or methionine) reacts with the L-amino acid oxidase and therefore the amount of L-amino acid determined is equivalent to the amount of dipeptide substrate hydrolysed.

Preparation of a cytoplasmic fraction from intestinal mucosa

As will be shown in Chapter 6, cytoplasmic peptide hydrolase activities are slightly, though not significantly, decreased if the intestine is removed from a freshly killed rat when compared with those of an intestine removed from an ether-anaesthetized rat. Therefore, intestines were removed from living, but anaesthetized, rats, except during the initial investigation of the assay when intestines were removed from ether killed rats.

The procedure for the preparation of an intestine for perfusion, described in Chapter 3, was followed and P.V.C. gloves were worn throughout to prevent contamination of the intestine during handling. Once the luminal contents had been washed out with warm (37°C) bicarbonate saline equilibrated with O_2/CO_2 (95:5), the whole jejunum plus ileum was torn away from the mesentery and excised just proximal to the ileo-caecal valve. Immediately, the intestine was placed on a piece of aluminium foil, pressed into a beaker of crushed ice to form a depression to hold the intestine, and taken to a cold room (4°C). Blood was removed from the serosal surface by rinsing the intestine in chilled NaCl solution (0.9 g NaCl in 100 ml distilled water). For ease of handling the intestine was divided into two; each piece was slit open with sharp scissors and laid flat, mucosal side uppermost, on a pre-chilled glass sheet resting on a tray of crushed ice. Two chilled glass microscope slides were used to scrape off the mucosa which was then transferred to a glass homogenizer vessel previously calibrated at a volume of 10 ml. The total volume of the mucosa was made up to 10 ml with chilled NaCl solution (0.9% w/v) and the sample was homogenised with a loose fitting 'Teflon' pestle. The pestle was rotated by a Gallenkamp 'Handilab Stirrer' operated at maximum speed (about 1200 rev/min). Homogenization was standardized to 30 strokes where 1 stroke represents movement of the pestle down through the homogenate and back up again. This took about 3 minutes to complete.

Finally, the homogenate was divided into portions. If required, two 2 ml portions were transferred to capped plastic tubes and stored at -40°C prior to estimation of the DNA content of the mucosa - details given in Chapter 5. A portion of the homogenate

(1 ml) was used for estimation of the total water content and the rest was transferred to a pre-chilled capped plastic centrifuge tube and centrifuged immediately in a pre-cooled centrifuge (M.S.E.) at 20 000 g at 4°C for 1 hour. The supernatant fraction was decanted into chilled plastic tubes on ice and was used immediately for the peptide hydrolase assay.

Peptide hydrolase assay

The following solutions were prepared freshly:

Tris-HCl buffer, 50 mmol/l (pH 8.4)	6.057 g Tris (hydroxymethyl) amino methane (Sigma London Chemical Co.) in 900 ml distilled water. The pH was adjusted to 8.4 with HCl (5 mol/l) and the volume was made up to 1 litre.
Tris-HCl buffer, 250 mmol/l (pH 7.8)	30.285 g Tris (hydroxymethyl) amino methane in 900 ml distilled water. The pH was adjusted to 7.8 with HCl (5 mol/l), and the volume was made up to 1 litre.
Gly-L-Met, 30 mmol/l	61.6 mg Gly-L-Met (Sigma London Chemical Co.) in 10 ml distilled water.
L-Leu-Gly, 30 mmol/l	56.5 mg L-Leu-Gly (Sigma) in 10 ml distilled water.
L-Val-L-Leu, 30 mmol/l	69.1 mg L-Val-L-Leu (Sigma) in 10 ml distilled water.

L-amino acid oxidase reagent 6 mg L-amino acid oxidase (Crotalus adamanteus venom, Sigma London Chemical Co. Type IV, purified),
1 mg peroxidase (Boehringer, purified) and 10 mg O-dianisidine (Sigma, water soluble) made up to a total volume of 100 ml with Tris-HCl buffer (250 mmol/l, pH 7.8).

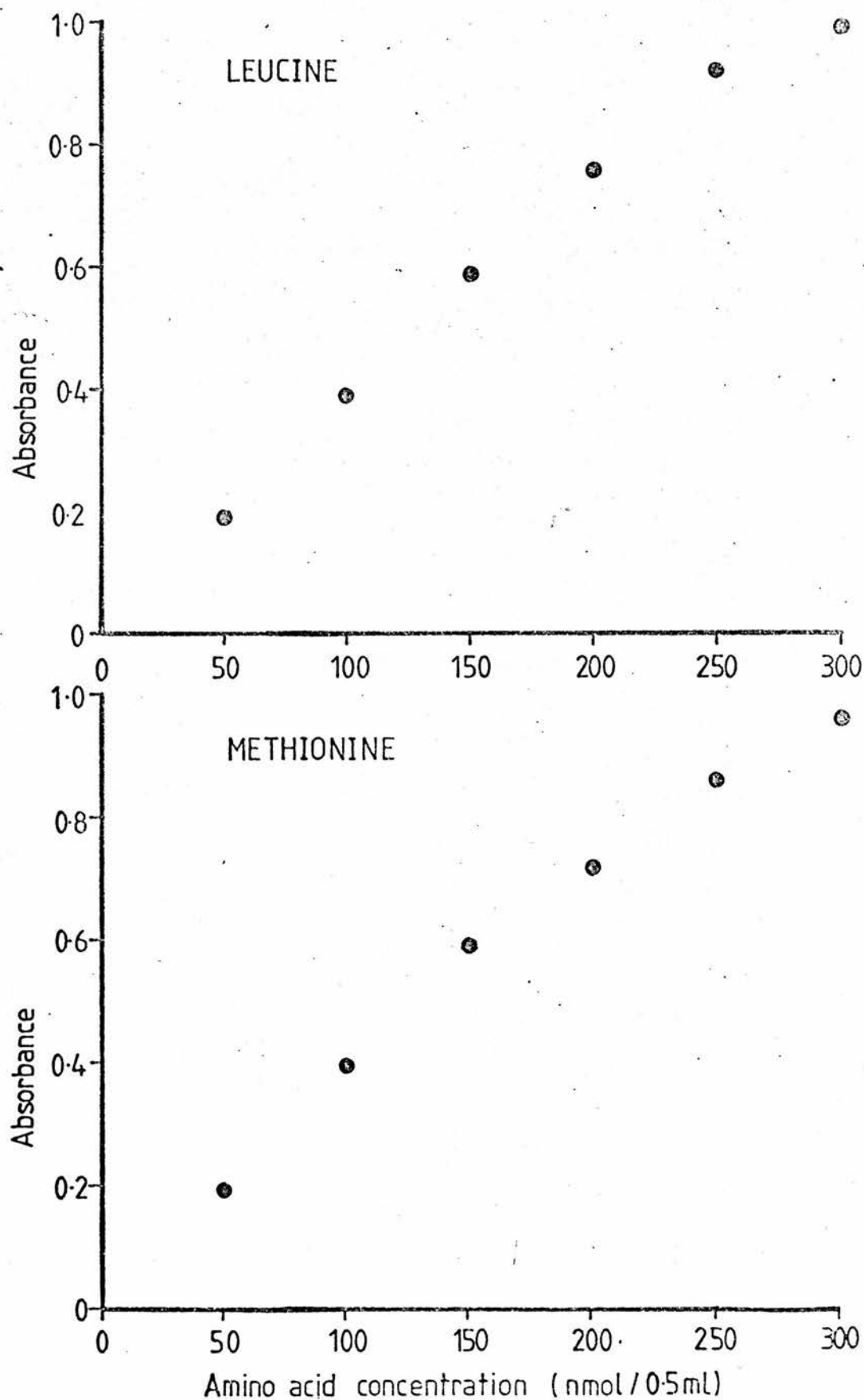
Procedure:

Peptide hydrolase activities against three dipeptide substrates, Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu, were estimated in duplicate at two suitable dilutions of the supernatant fraction to be assayed.

The supernatant fraction was diluted in the range 1:320 to 1:1280 in Tris-HCl buffer (50mmol/l) pH 8.4. To tubes containing 0.4 ml dipeptide substrate, 0.1 ml of a supernatant dilution was added. Blanks were prepared containing either 0.4 ml dipeptide substrate and 0.1 ml Tris-HCl buffer (50mmol/l) pH 8.4, 0.4 ml (50 mmol/l) Tris-HCl buffer pH 8.4 and 0.1 ml of the supernatant dilution, or 0.5 ml Tris-HCl buffer (50mmol/l) pH 8.4. All tubes were mixed rapidly with a vortex mixer and then incubated in a water bath at 37°C for 30 minutes. After incubation the tubes were transferred to a boiling water bath for 3 minutes to terminate the reaction and then removed and allowed to cool to room temperature. 1 ml of the L-amino acid oxidase reagent was then added to the sample tubes which were mixed and incubated for 1 hour at room temperature.

Sample absorbance was measured within 2 hours in a Pye Unicam

Fig. 4.2 Calibration curves for the assay of L-Leucine and L-Methionine with L-amino acid oxidase from snake venom by the method used by Gardner et al (1978). Each point is the mean of 2 estimations.



SP1750 spectrophotometer at 440 nm. All samples were measured against the Tris-HCl buffer blank. The absorbance measurements were corrected for the absorbance, if any, of the supernatant and dipeptide blanks.

Calibration curve

A calibration curve was prepared for each set of estimations. Standard amino acid solutions, 500 nmol/l (26.24 mg L-Leucine or 29.84 mg L-Methionine in 200 ml distilled water, stored at -40°C in 2 ml portions) were diluted in the range 100 - 300 nmol/l in Tris-HCl buffer (50mmol/l, pH 8.4). 1 ml of the amino acid oxidase reagent was added and the samples were incubated as above.

The calibration curve was almost linear over this range (Fig. 4.2) and the amino acid content of the test samples was determined from the first order regression line of the calibration curve for the relevant amino acid.

Estimation of the total water content of the homogenate

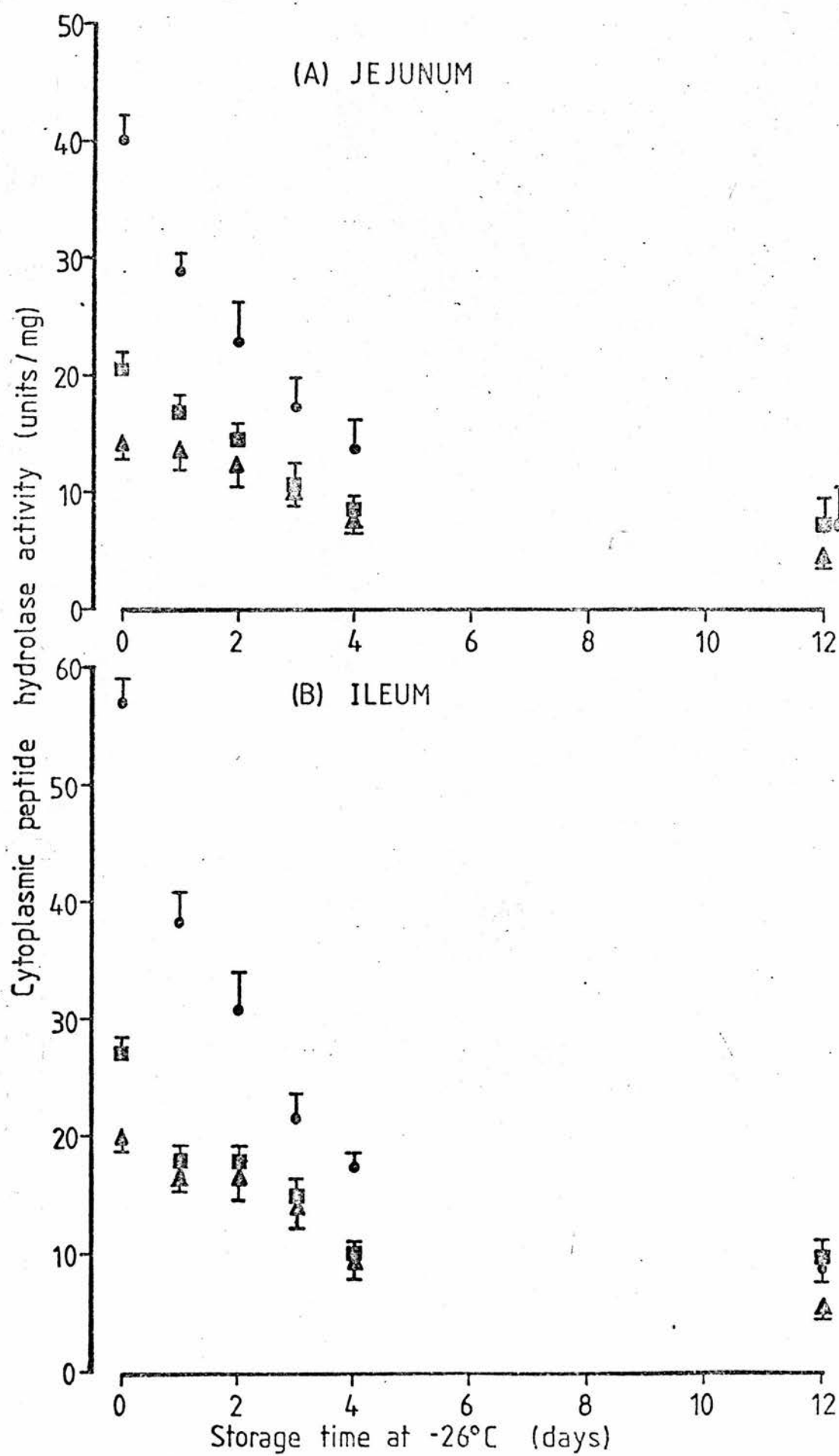
The total water content of the supernatant was calculated from the difference between the wet and dry weight of the homogenate. A 1 ml portion of the homogenate was pipetted into a pre-weighed plastic dish and the wet weight was recorded. The sample was dried to constant weight at 37°C in an incubator and the water content was calculated from the weight loss. The cytoplasmic enzyme activities were assumed to be evenly distributed throughout the water volume of the homogenate. Activities estimated per ml of supernatant were multiplied by the total supernatant volume to give the total activity in the supernatant.

The effect of storage of the supernatant on peptide hydrolase activities

It was important to determine whether the supernatant fraction could be stored frozen and used subsequently for the estimation of peptide hydrolase activities. Segments (about 1 cm) of proximal jejunum and distal ileum were removed from an anaesthetised rat after the jejunum plus ileum had been removed for perfusion measurements. The intestinal mucosa was scraped off as described previously, and homogenized in 2 ml of NaCl solution (0.9% w/v). The homogenates were transferred to pre-chilled centrifuge tubes and centrifuged at 20 000 g for 1 hour at 4°C. A portion of the supernatant was taken for immediate estimation of peptide hydrolase activities and the rest was stored frozen at -26°C (the estimated temperature of the deep freeze) in 0.1 ml portions in capped plastic tubes. Peptide hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu were estimated on each of the next 4 days and on the twelfth day after preparation of the supernatant. A single 0.1 ml portion was thawed for use each day. Activities were expressed with reference to protein (estimated by the method of Lowry et al, 1951). The results are shown in Fig. 4.3.

There was a dramatic decrease ($P < 0.001$) in all three activities after storage for 12 days at -26°C and the decrease was greatest for activities against Gly-L-Met (84%). Furthermore, all activities were decreased after storage at -26°C for only 1 day although the decrease was only significant for activities against Gly-L-Met (27% for the jejunum, 33% for the ileum $P < 0.001$) and L-Val-L-Leu (18% for the jejunum, 36% for the ileum $P < 0.001$).

Fig. 4.3 Cytoplasmic peptide hydrolase activities against (●) Gly-L-Leu, (▲) L-Leu-Gly, and (■) L-Val-L-Leu estimated on the mucosa from (A) jejunum and (B) ileum on the day of preparation of the supernatant fraction and after storage at -26°C for various times. Activities are expressed per mg protein. Values are the mean \pm S.E.M. of observations on 4 intestines.



The changes were similar for activities in the jejunum and ileum.

Thus it is clear that cytoplasmic peptide hydrolase activities are decreased if the supernatant is stored frozen overnight prior to estimation of the activities: hence activities should be estimated immediately after preparation of the supernatant.

Application of the assay to the estimation of peptide hydrolase activities in mucosal cytoplasm, serosal secretions and luminal effluents

This method was used in the study of the release of peptide hydrolase activities from the rat small intestine during perfusion, described in Chapter 6. However, activities could not be estimated with any degree of accuracy in the luminal effluents and serosal secretions since these activities were at the lower limit of the sensitivity of the assay ($0.02 \mu\text{mol}/\text{min}/\text{ml}$). Furthermore, estimates of cytoplasmic peptide hydrolase activities in the intestinal mucosa varied to an unsatisfactory extent between duplicate samples (coefficient of variation of an estimate = 12.3%, $n = 10$).

Peptide hydrolase activities estimated on different dilutions of the supernatant fraction

Fig. 4.4 shows the time course of the hydrolase activity against L-Leu-Gly estimated at three different dilutions of the supernatant fraction, 1:160, 1:320 and 1:640. The assay was performed as described previously except that the first incubation time was varied from 5 to 60 minutes. The amount of L-Leucine released is expressed per ml of supernatant.

It is clear that the progress curve of the reaction is non-linear

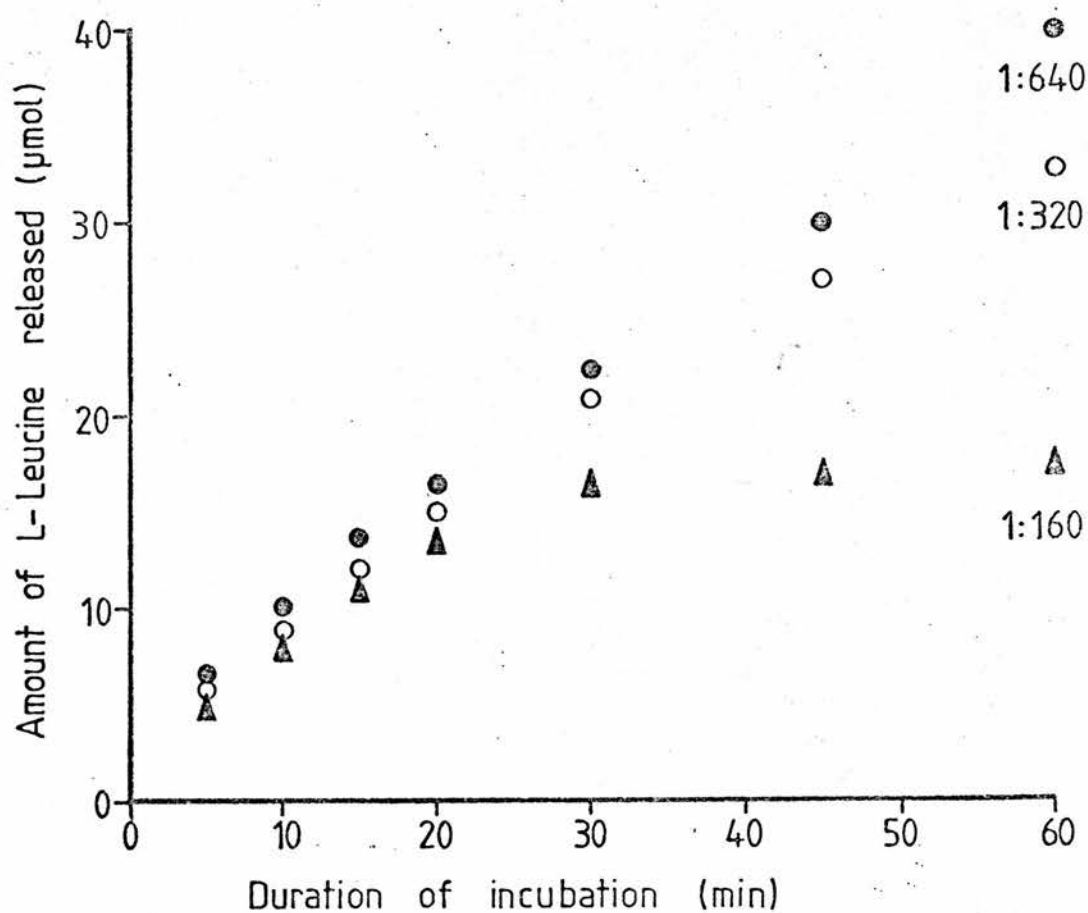


Fig. 4.4 The amount of L-Leucine released after incubation of the supernatant fraction of a mucosal homogenate in the presence of 15 mmol/l L-Leu-Gly. The supernatant was used at three dilutions; 1:160 (Δ), 1:320 (○) and 1:640 (●). The amount of L-Leucine released is expressed per ml of undiluted supernatant. Each point is the mean of 3 estimations.

for all three dilutions used; there is, however, a trend towards linearity with increasing dilution of the activities. This effect was found to be due partly to the low solubility of O-dianisidine in the pH 7.8 Tris buffer and partly to the non-linearity of the assay at amino acid concentrations greater than 150 nmol/0.5 ml (Fig. 4.2). However, provided that the O-dianisidine was completely dissolved in 1 ml of distilled water prior to addition of the Tris buffer (as recommended by Fujita et al, 1972), this effect was reduced.

DISCUSSION OF METHOD '1'

The method of Gardner et al (1978) lacked sufficient sensitivity to enable the estimation of peptide hydrolase activities in the luminal effluents and serosal secretions from perfused intestines. Furthermore, the method was found to have several shortcomings which were introduced when the method was modified from that of Fujita et al (1972). The calibration curves were non-linear over the range used by Gardner et al (1978) and this resulted in low estimates of peptide hydrolase activities at low dilutions of the supernatant fraction. This effect was emphasised by the insolubility of the chromogen, O-dianisidine, in the Tris buffer; the extent of solubility also varied between batches of the L-amino acid oxidase reagent.

Although the latter shortcomings of the method could be easily overcome, its lack of sensitivity made it unsuitable for use in this work. Furthermore, Lindberg et al (1975) have rightly criticised the technique of boiling the sample in order to inactivate the peptide hydrolase activities since this may temporarily speed up the reaction. Therefore other methods were investigated.

Method '2': The method of Nicholson and Kim (1975)

The method of Nicholson and Kim (1975) is based on the same principle as that of Fujita *et al* (1972). However, Nicholson and Kim have combined the two incubations into a single incubation and the whole process is terminated by the addition of sulphuric acid. This produces a purple complex which is stable overnight as opposed to the brown colour resulting from the oxidation of O-dianisidine which is stable for only about 2 hours (Fujita *et al*, 1972). This method was therefore investigated and the results were compared with those obtained by a similar method, also reported by Nicholson and Kim (1975), in which the two incubations were performed separately in order to determine whether the L-amino acid oxidase inhibits the peptide hydrolase activities. These are referred to below as the 'one-step' and 'two-step' assays.

The 'one-step' peptide hydrolase assay of Nicholson and Kim (1975)

This method was investigated and was found to give consistently lower activities than were observed with the 'two-step' assay even with the dipeptide substrates used by Nicholson and Kim (1975) (i.e. L-Leu-L-Ala, L-Phe-Gly and Gly-L-Phe, results not shown). Addition of L-amino acid oxidase to the first of the two incubations of the 'two-step' assay resulted in a similar reduction in the activity to that observed when activities were estimated by the 'one-step' method. Addition of peroxidase or O-dianisidine to the first of the two incubations had no effect on the activities estimated by the 'two-step' assay.

It was therefore concluded that the peptide hydrolase activities were inhibited by the L-amino acid oxidase and that the combination



of the two incubations is not possible for these activities. However, the second method reported by Nicholson and Kim (1975) involves two incubations, each of 20 minutes, and is therefore quicker to perform than that of Fujita *et al* (1972). The method is also more sensitive since the lower limit of sensitivity is about 6 nmol/min/ml. This second 'two-step' method was therefore modified for use in this work.

The 'two-step' peptide hydrolase assay of Nicholson and Kim (1975)

The following solutions were prepared freshly:

Tris-HCl buffer, 50 mmol/l (pH 8.0)	6.057 g Tris (hydroxymethyl) amino methane in 900 ml distilled water. The pH was adjusted to 8.0 with HCl (5 mol/l), and the volume was made up to 1 litre.
Gly-L-Met, 20 mmol/l	41.26 mg Gly-L-Met in 10 ml Tris-HCl buffer.
L-Leu-Gly, 15 mmol/l	28.23 mg L-Leu-Gly in 10 ml Tris-HCl buffer.
L-Val-L-Leu, 15 mmol/l	34.55 mg L-Val-L-Leu in 10 ml Tris-HCl buffer.
L-amino acid oxidase reagent	20 mg L-amino acid oxidase, 2 mg peroxidase and 10 mg O-dianisidine (dissolved in 1 ml distilled water) were dissolved in 100 ml Tris-HCl buffer.

(The source of the chemicals was as stated previously).

Procedure:

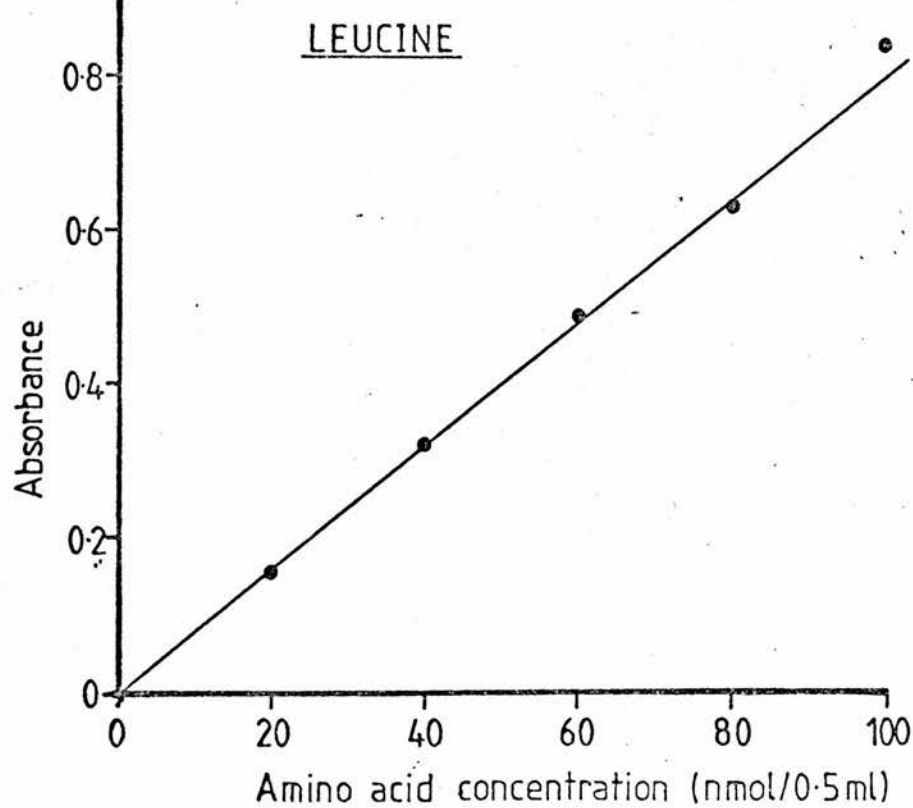
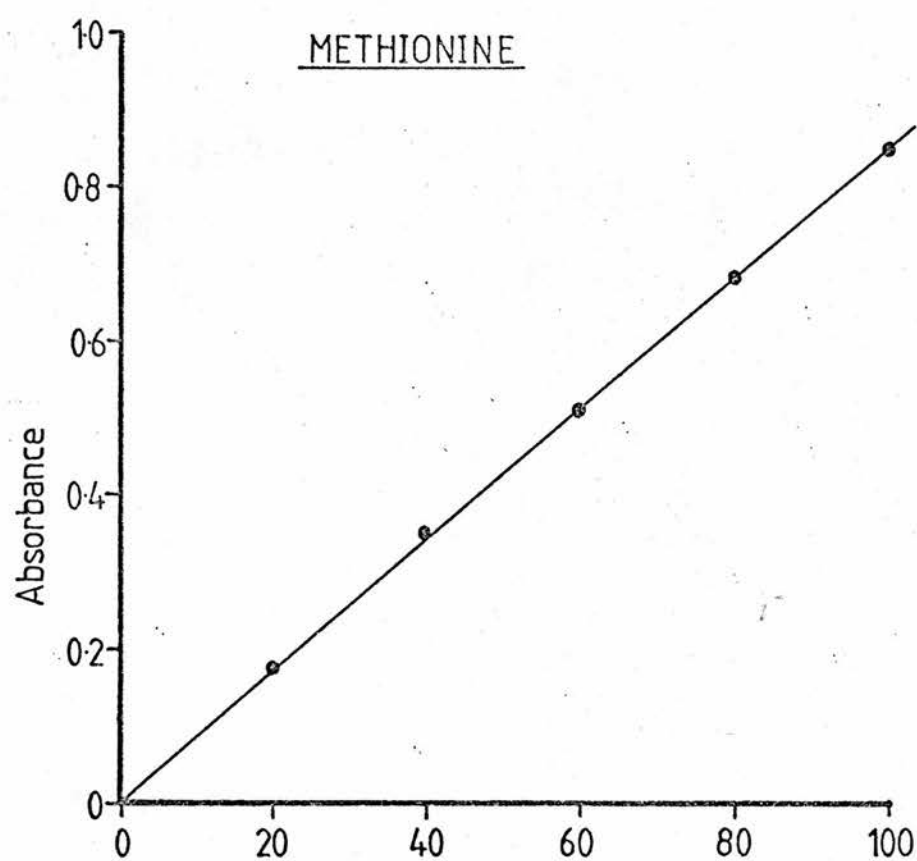
A supernatant fraction was prepared as described previously. In order to minimise thermal inactivation of the enzyme activities they were estimated immediately after centrifugation of the homogenate.

Samples were diluted in the range 1:320 to 1:2560 by serial dilution on ice (4°C) in pre-chilled Tris-HCl buffer (50mmol/l, pH 8.0). Although Nicholson and Kim (1975) used 14% glycerol for the dilution of their homogenate this was omitted since glycerol was found to inhibit all three activities estimated.

Activities against three dipeptide substrates, Gly-L-Met, L-Leu-Gly and L-Val-L-Leu, were estimated in duplicate at each of two dilutions. Tubes containing 0.5 ml dipeptide substrate were placed in a water bath at 37°C and 0.04 ml of a suitable dilution of the supernatant, luminal effluent or serosal secretion was added. Samples were mixed rapidly with a vortex mixer and incubated for 20 minutes at 37°C . The samples were removed from the water bath and placed in a boiling water bath for 6 minutes to terminate the peptide hydrolysis reaction. After cooling to room temperature the tubes were returned to the 37°C water bath and 1 ml of the L-amino acid oxidase reagent was added. The samples were shaken and incubated for 20 minutes at 37°C after which 0.74 ml of 50% H_2SO_4 ($\text{H}_2\text{SO}_4 : \text{H}_2\text{O}$ 1:1) was added and the tubes were removed from the water bath and allowed to cool to room temperature.

The absorbance was measured at 530 nm in a Pye Unicam SP1750 spectrophotometer read against a blank consisting of 0.54 ml Tris-HCl buffer (50mmol/l, pH 8.0). 1 ml L-amino acid oxidase reagent and

Fig. 4.5 Calibration curve for the assay of L-Leucine and L-Methionine with L-amino acid oxidase from snake venom by the 'two-step' method of Nicholson and Kim (1975). Each point is the mean of 2 estimations.



0.74 ml 50% H_2SO_4 .

Calibration curve

A calibration curve was prepared for each set of estimations in the range 20 - 100 nmol L-Leucine or L-Methionine in 0.5 ml Tris-HCl buffer (50mmol/l, pH 8.0). The samples were incubated for 20 minutes at 37°C with 1 ml L-amino acid oxidase reagent and then 0.74 ml of 50% H_2SO_4 was added. The absorbance was read as above.

The calibration curve was linear over this range (Fig. 4.5). The amount of dipeptide substrate hydrolysed was equivalent to the amount of free amino acid estimated and this was determined for the test samples from the first order regression line of the calibration curve.

Sample blanks

Blanks were included in all estimations, consisting of 0.5 ml of Tris buffer and 0.04 ml of each supernatant dilution. The absorbance of the test samples was corrected for the absorbance of the blank, although this was normally negligible.

The optimal substrate concentrations

Peptide hydrolase activities were estimated on a suitable dilution of a supernatant fraction with a range of dipeptide substrate concentrations (5 - 30 nmol/l). The results are shown in Fig. 4.6. All three activities estimated, Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu, were inhibited by high concentrations of the dipeptide substrate. The optimal substrate concentration was therefore determined as the substrate concentration which gave the highest estimate

Fig. 4.6 Cytoplasmic peptide hydrolase activities against Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu estimated at various dipeptide substrate concentrations. Activities are expressed as total units per ml of supernatant. Each point is the mean of 3 estimations.

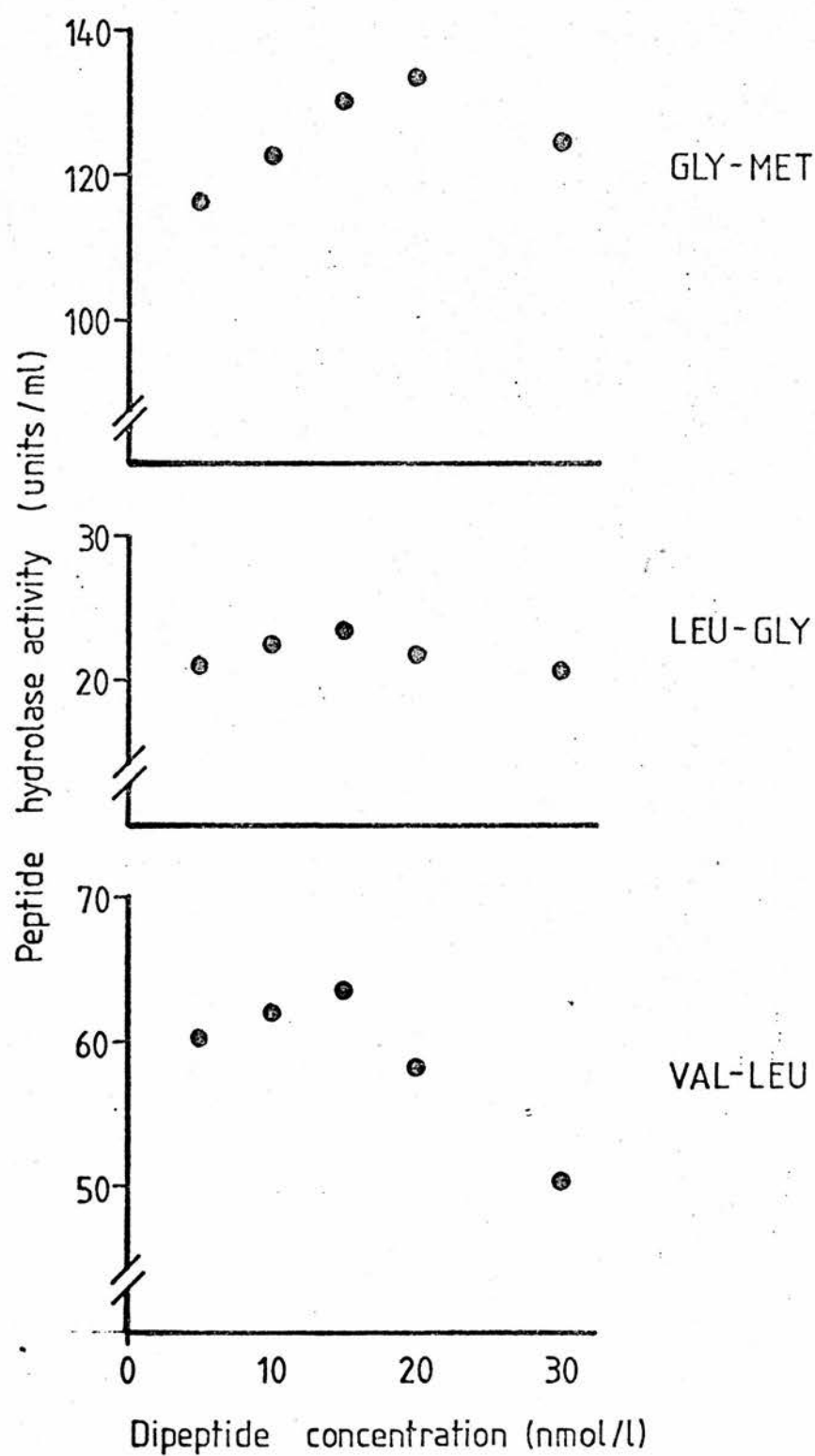
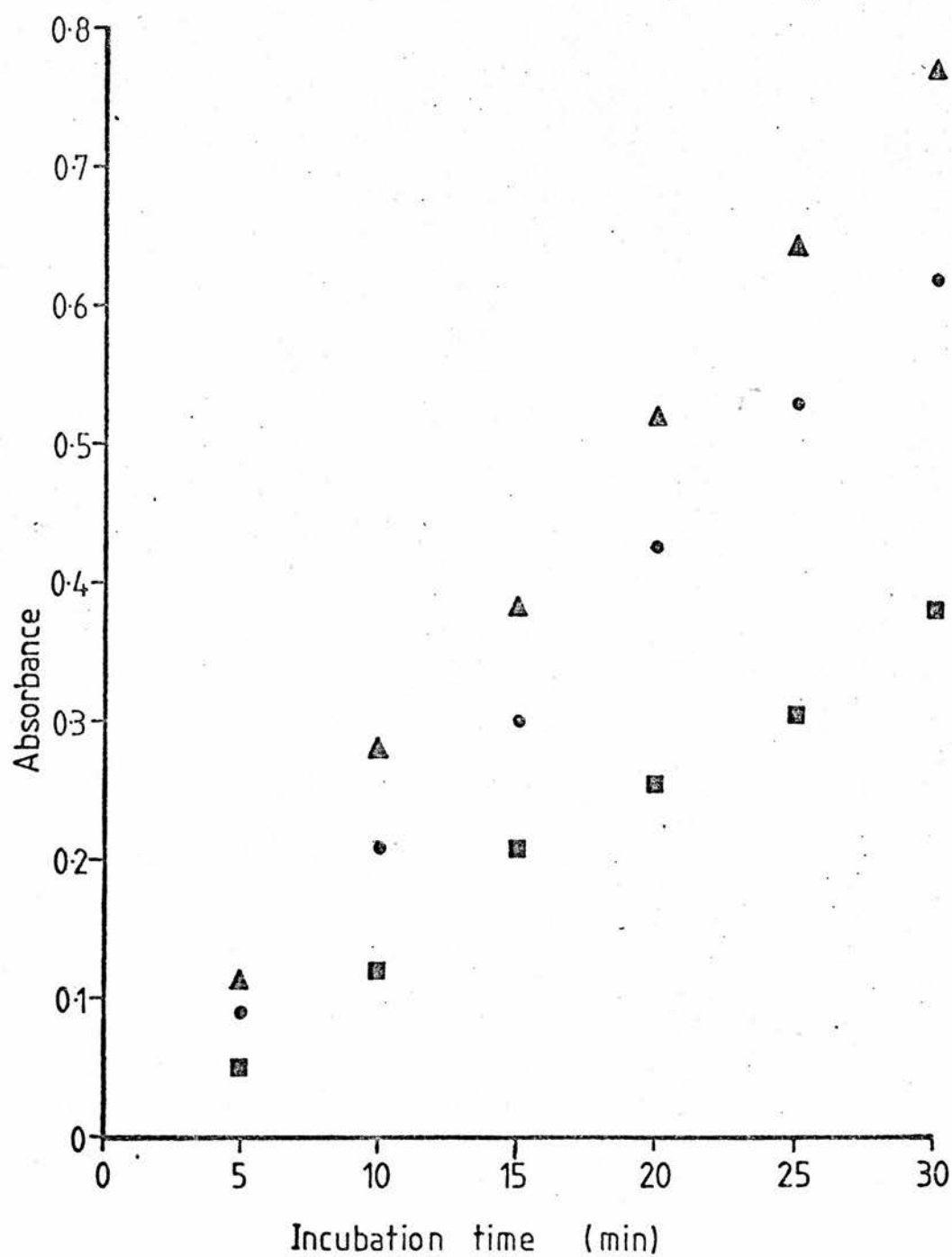


Fig. 4.7 The amount of free amino acid estimated with L-amino acid oxidase after incubation for various times of a suitable dilution of a supernatant fraction from a mucosal homogenate in the presence of either Gly-L-Met (20 mmol/l) (●), L-Leu-Gly (15 mmol/l) (■), or L-Val-L-Leu (15 mmol/l) (▲). The absorbance of the samples are plotted against the incubation time and each point is the mean of 3 estimations.



of peptide hydrolase activities: these were Gly-L-Met - 20 nmol/l, L-Leu-Gly - 15 nmol/l and L-Val-L-Leu - 15 nmol/l.

Progress curve of the reaction

The amount of free amino acid released was estimated with the L-amino acid oxidase reagent after incubation for various times at 37°C of a suitable dilution of a supernatant fraction in the presence of either Gly-L-Met (20 nmol/l), L-Leu-Gly (15 nmol/l) or L-Val-L-Leu (15 nmol/l). The results are shown in Fig. 4.7.

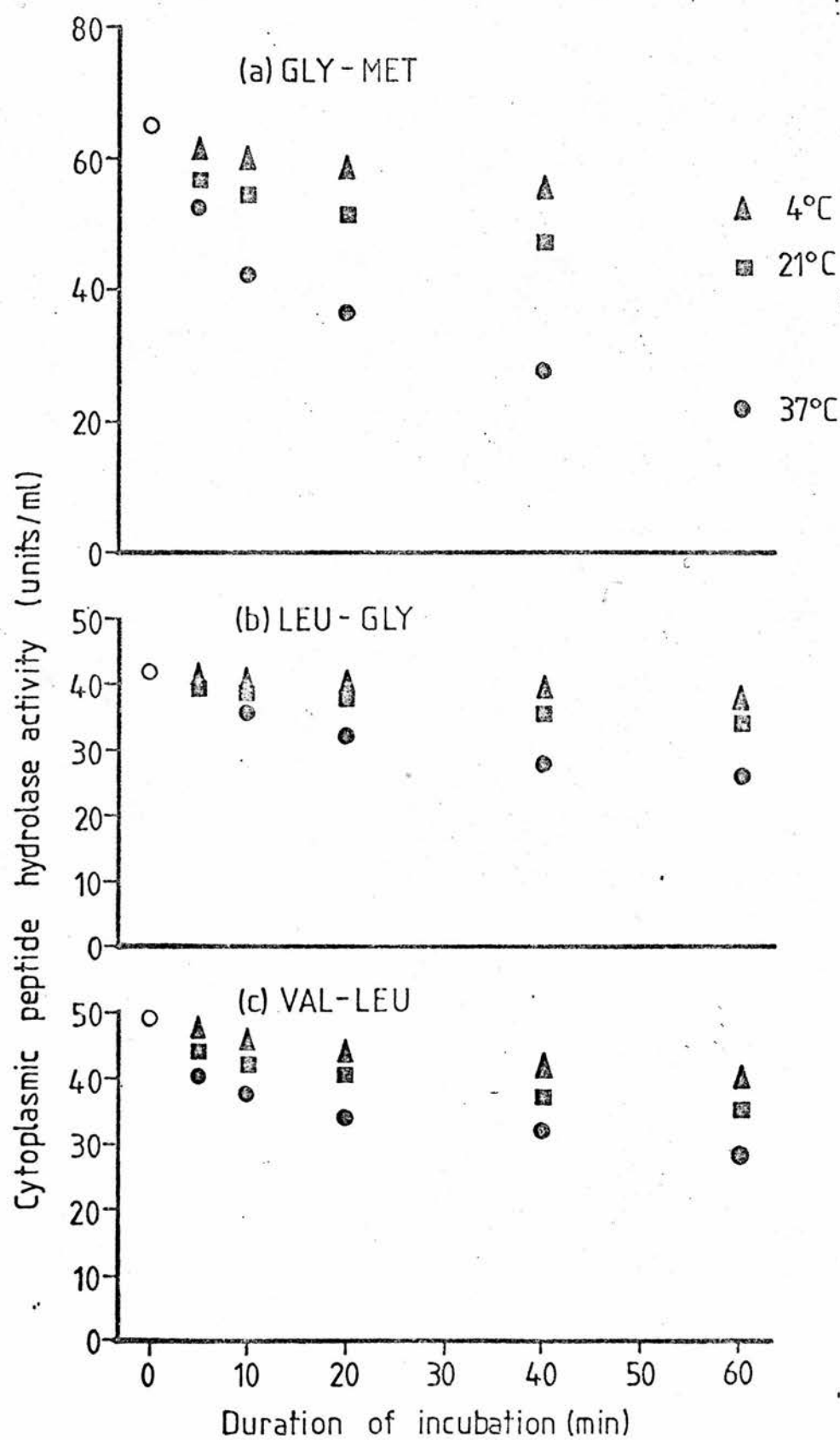
The reaction is linear with time for at least the first 30 minutes for all three activities estimated. It is therefore justifiable to use a single point estimate at 20 minutes to measure peptide hydrolase activities.

Thermolability of the peptide hydrolase activities

A supernatant fraction prepared from a mucosal homogenate was divided into four portions. Peptide hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu were estimated immediately on one portion and then at various times either after storage on ice in a refrigerator at 4°C or after incubation at 21°C or 37°C in a water bath. The results are shown in Fig. 4.8.

Activities against all three dipeptide substrates decreased with time. However the decrease was greatest, by 66%, 49% and 37% for activities against Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu respectively, when the enzymes were incubated for 1 hour at 37°C and least (19%, 18% and 11% respectively) when the supernatant fractions were stored at 4°C for 1 hour. Furthermore, incubation

Fig. 4.8 Cytoplasmic peptide hydrolase activities against (a) Gly-L-Met, (b) L-Leu-Gly, and (c) L-Val-L-Leu estimated at various times after incubation of the supernatant fraction at (Δ) 4°C, (\blacksquare) 21°C, and (\bullet) 37°C. Activities estimated immediately after preparation of the supernatant fraction are also shown (O). Each point is the mean of 3 estimations.



for only 5 minutes at 21°C (room temperature) resulted in a decrease by 12%, 5% and 10% of activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu respectively.

These results show clearly that it is essential to maintain strict control of temperature at 4°C during preparation of samples containing peptide hydrolase activities and, furthermore, that these activities should be estimated immediately after preparation of the supernatant fraction (or after collection of the luminal effluent and serosal secretion from a perfused intestine).

DISCUSSION

The two methods of Nicholson and Kim (1975) have been investigated. Although the combination of the two reactions, hydrolysis of the dipeptide substrate and determination of the released amino acid with L-amino acid oxidase, was found to be unsatisfactory the separation of the reactions into two 20 minute incubations proved to be a highly sensitive and satisfactory method for the estimation of peptide hydrolase activities. This method has been used to estimate these activities in the mucosal cytoplasm of the small intestine and in the luminal effluents and serosal secretions from a perfused small intestine.

Lindberg et al (1975) criticised the use of the amino acid oxidase reaction for the estimation of peptide hydrolase activities since the enzyme preparation itself may contain amino acids and since termination of the peptide hydrolase activity against the dipeptide substrate by boiling the samples might increase the reaction rate for a short period. The first criticism was found to be unjustified since enzyme blanks were included in estimations and these were, without exception, found to contain very little amino acid relative to the amounts released during the assay. The second point is a justified criticism of the method. However, it is stressed that the assay was used only in comparative studies and therefore this source of error should not affect the overall conclusions.

It is clear that all three enzymic activities estimated in this work are unstable and that the instability is increased with increased storage temperature. In order to minimise the thermolability of the enzymes strict control of temperature during

preparation of the samples has been introduced and the activities were always estimated immediately after preparation of the samples.

It is not known whether the three activities assayed in this work represent three individual enzymes or three activities of a single enzyme. Although hydrolase activities against a large number of dipeptide substrates have been demonstrated to be present in the intestinal mucosa (see Lindberg *et al*, 1975, Table 6.1) it is not known whether there are a large number of dipeptidases with a narrow specificity or a small number of dipeptidases with a broad specificity. There have been several reports of multiple forms of dipeptide hydrolase activities which can be separated by starch gel electrophoresis (Dolly *et al*, 1971; Fottrell *et al*, 1972; Kim *et al*, 1971). However, Das and Radhakrishnan (1973) reported an electrophoretically homogenous dipeptidase activity, purified from the soluble extracts of monkey small intestine, which was shown to hydrolyse at least 65 dipeptide substrates. They suggested that there is a 'master' dipeptidase in the small intestine and possibly only four or five other dipeptidases.

The 'master' dipeptidase of Das and Radhakrishnan (1973) can hydrolyse all three substrates used in this work and it is possible, therefore, that only one enzyme has been studied. However, there is some evidence from the results presented in Chapters 6 and 10 and here which suggests tentatively that at least two enzymes have been studied:

(1) In Chapter 6 it is shown that the activities against all three dipeptide substrates (Gly-L-Met, L-Leu-Gly and L-Val-L-Leu) appearing in the luminal effluent during perfusion *in vitro* are greater when the intestine is removed from a freshly killed rat than

when the intestine is removed from an ether-anaesthetized rat. However, there was a much greater increase in activities against Gly-L-Met and L-Val-L-Leu (8 fold) than in activities against L-Leu-Gly (4 fold).

(2) There was a slight increase (11%) in peptide hydrolase activities against Gly-L-Met when rats were fed for 1 week on an 'elemental' diet, 'Flexical', whereas activities against L-Leu-Gly and L-Val-L-Leu were decreased by a similar amount (Chapter 10, Fig. 10.1). Gardner and Heading (1979) made a similar observation on rats fed on casein-based diets, although, in their work, the decreases in activity against L-Val-L-Leu and L-Leu-Gly were not significant.

(3) Furthermore, all three activities are thermolabile (Fig. 4.7) but this is much more marked for activities against Gly-L-Met than against L-Leu-Gly and L-Val-L-Leu.

These three observations suggest that Gly-L-Met might be a separate enzymic activity but there is very little evidence to suggest that the activities against L-Leu-Gly and L-Val-L-Leu are due to more than one enzyme.

Although this question is of interest, its solution was not central to the intended use of the peptidase assays in the present project, and therefore it was not pursued.

In view of the satisfactory outcome of the investigation of the 'two-step' method of Nicholson and Kim (1975) the method as detailed above was adopted for the work described in Chapters 6 and 10 of this thesis.

CHAPTER 5

Estimation of DNA in intestinal mucosa

Introduction

Many methods for determination of nucleic acids in biological materials that involve colorimetry or ultraviolet spectrophotometry require separation of the nucleic acids from free nucleotides and other interfering substances; they also lack sensitivity (Munro and Fleck, 1966). LePecq and Paoletti (1966) developed a fluorometric method for the determination of purified nucleic acid based on the enhancement of the fluorescence of the dye 2,7-diamino-9-phenylphenanthridine 10-ethyl bromide (ethidium bromide) due to complex formation with native nucleic acid. Prasad *et al* (1972) have modified the method to permit the measurement of nucleic acids in tissue homogenates. Batt and Peters (1976) have already applied this method successfully to the estimation of cell numbers in intestinal homogenates, and the simplicity of the method made it an obvious choice for use in the estimation of the DNA content of the intestinal mucosa.

Principle of the assay

Ethidium bromide is added to a buffered sample of tissue homogenate containing 5 µg or less of DNA. The buffer is added to maintain the pH and salt concentration such as to minimise nucleic acid-protein interactions. The fluorescence of this solution is due to the presence of both DNA and RNA. The RNA is removed by incubation with RNAase and the fluorescence due to DNA alone is measured. The fluorescence due to RNA can be derived from the difference between the first and second fluorescence readings.

Preparation of an homogenate of intestinal mucosa

The method of preparation of a mucosal homogenate was as described in Chapter 4 except that the samples were stored for up to 5 hours at 4°C prior to estimation of the DNA content. P.V.C. gloves were worn throughout in order to minimize contamination of the sample with exogenous nuclease activities.

DNA estimation - Preliminary trials

The following solutions were prepared just before use:

Tris buffer, 0.1 mmol/l	12.11 g Tris (hydroxymethyl) amino methane and 5.84 g NaCl (both from Sigma London Chemical Co.) in 900 ml distilled water and adjusted to pH 8.0 with HCl (5 mol/l), and the volume made up to 1 litre.
Ethidium bromide	2 mg ethidium bromide (Sigma London Chemical Co.) in 100 ml distilled water.
DNA	A stock solution of DNA (Calf thymus, sodium salt, Type I, Sigma London Chemical Co.) was prepared by addition of 100 ml of 0.9% NaCl solution (0.9g NaCl in 100 ml distilled water) to 5 mg of DNA. This was stirred by a magnetic stirrer for 4 hours at 4°C (in a cold room).

RNA

A stock solution of RNA (Calf liver, Type IV, Sigma London Chemical Co.) was prepared by addition of 100 ml 0.9% NaCl solution to 10 mg RNA.

RNAase

20 mg RNAase (Ribonuclease A type 111A from bovine pancreas, Sigma London Chemical Co.) dissolved in 1 ml distilled water.

The mucosal homogenate was mixed well with a vortex mixer. A portion (0.1 ml) was diluted at room temperature in the range 1:80 to 1:640 by serial dilution in the Tris buffer (0.1mol/l). Sample tubes were prepared containing 0.2 ml diluted homogenate, 1.8 ml Tris buffer and 2 ml ethidium bromide solution. These were mixed with a vortex mixer and the fluorescence was read in a Perkin-Elmer 203 Fluorescence Spectrophotometer at an excitation wavelength of 365 nm and an emission wavelength of 590 nm. Zero emission intensity was set with a blank containing 2 ml Tris buffer and 2 ml ethidium bromide solution and 100% emission intensity was calibrated with a standard sample containing 10 μ g DNA. RNAase (0.04 ml) was then added to all tubes and they were mixed well and incubated in a water bath at 50°C for 1 hour. After removal from the water bath the samples were cooled to room temperature prior to measurement of the fluorescence due to DNA alone.

Calibration curve

A calibration curve was prepared in the range 10 - 50 μ g DNA/ml and 10 - 60 μ g RNA/ml.

Estimation of the DNA content of a mucosal homogenate

Three major problems were encountered when the assay was applied to a mucosal homogenate:

(1) Fluorescence readings were very unstable especially at low dilutions of the homogenate.

(2) The results obtained with different dilutions of the homogenate did not agree.

(3) The RNAase digestion was very unreliable; frequently the first and second fluorescence measurements were similar.

The first two problems were both due to the particulate nature of the homogenate. Addition of the detergent 'Triton X100' (Sigma) had no effect on the stability of the readings. When the homogenate was boiled for 3 minutes to denature the proteins present and then rehomogenised the sample fluorescence readings were stable but the technique resulted in a reduction, by about 7%, of the estimated mucosal DNA content. Both problems were eventually solved by alteration of the technique used for serial dilution of the homogenate. Initially, samples were mixed with a vortex mixer prior to pipetting. However, when samples were mixed by repeated uptake and release from a 1 ml automatic pipette fluorescence measurements were very stable and good agreement was obtained between different dilutions.

RNA digestion

When test samples, prepared from a mucosal homogenate, were incubated for 1 hour at 50°C in the absence of RNAase the decrease in sample fluorescence was frequently similar to that observed in samples incubated in the presence of RNAase. Fig. 5.1 shows the

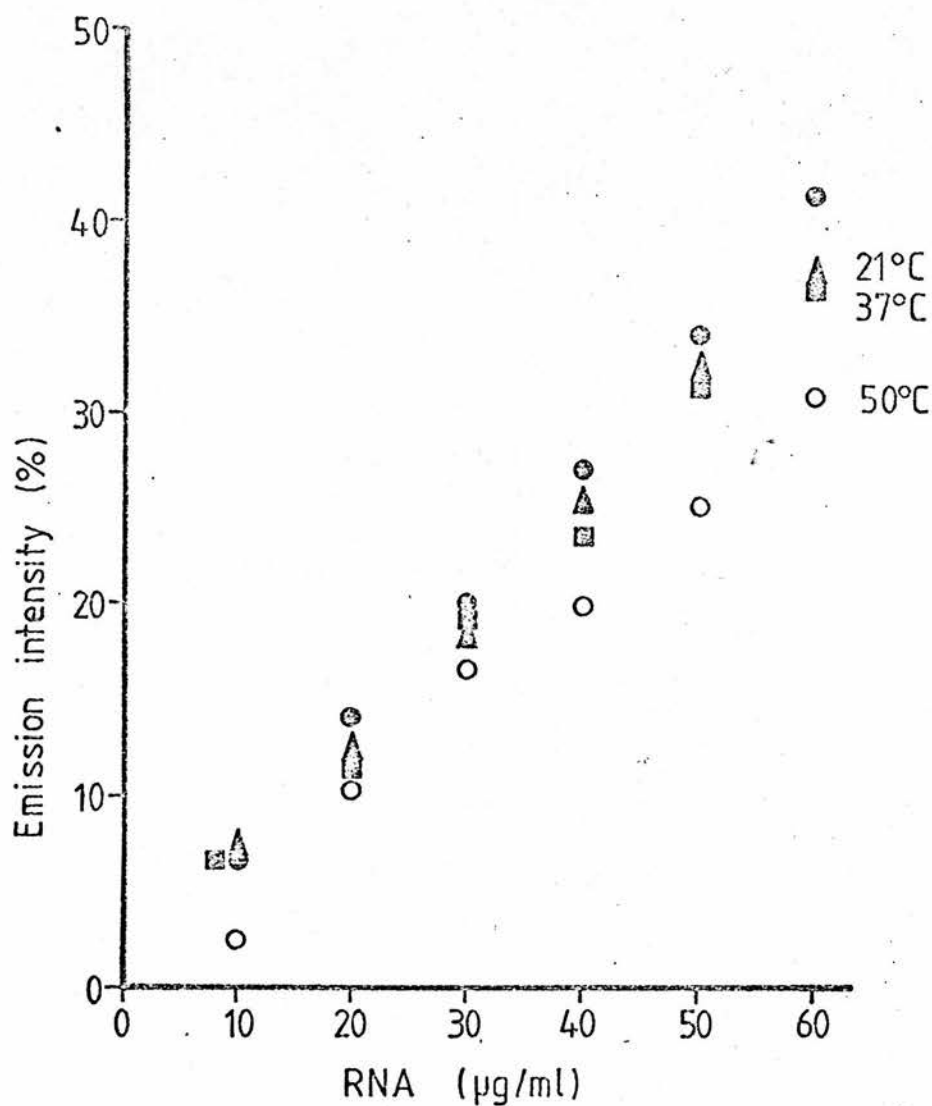


Fig. 5.1 Calibration curve for the estimation of RNA with ethidium bromide (●). Also shown is the emission intensity of the samples after incubation for 1 hour at 21°C (▲), 37°C (■) or 50°C (○). Each point is a single observation.

effect of incubation for 1 hour at 21°C, 37°C or 50°C on the fluorescence of RNA calibration standards. Sample fluorescence was decreased, by 3 - 8%, after incubation and the decrease was greatest for samples incubated at 50°C. Furthermore, incubation of DNA at 50°C for 1 hour in the presence of RNAase resulted in a 5% decrease in the fluorescence of the DNA sample (results not shown). Thus both RNA and DNA contents are decreased after incubation for 1 hour at 50°C.

An incubation temperature of 50°C for RNAase was recommended by Van Dyke and Szuslkiewicz (1968) to ensure the total removal of RNA from mixtures of RNA and DNA. Since at this temperature a small loss of DNA occurs the incubation temperature was decreased to 37°C. At this temperature DNA was stable for at least 1 hour in the presence of RNAase. The fluorescence enhancement of ethidium bromide by the most concentrated RNA standard (60 µg/ml) was reduced to less than 2%, and that of the other solutions to less than 1%, by incubation for 1 hour at 37°C in the presence of RNAase. This was acceptable since the RNA content of the mucosal homogenate dilutions was always less than 40 µg/ml.

DNA content of the intestinal mucosa

The assay was used to estimate the DNA content of the mucosa from the whole jejunum plus ileum. The mean value from 5 rats was 19.42 ± 1.53 mg/intestine. This is much lower, by about 41%, than the values quoted in the literature (see Table 5.1, p80).

In view of the fact that the incubation temperature had been reduced and the fact that the estimates of the mucosal DNA content appeared to be lower than those reported elsewhere alternative methods

were investigated.

The method of Karsten and Wollenberger (1972)

Karsten and Wollenberger (1972) have also developed the method of LePecq and Paoletti (1966) for application to the measurement of nucleic acids in tissue homogenates. The method of Prasad et al (1972) relies on the pH and salt concentration of the buffer to reduce nucleic acid - protein interactions and does not attempt to inhibit endogenous nuclease activity apart from stressing that the assay should be carried out rapidly to prevent this activity. In contrast, Karsten and Wollenberger (1972) include a protease during tissue homogenization and during the DNA assay. The activity of the protease, extracted from Streptomyces griseus, has two functions. It inactivates endogenous nucleases although it does not inhibit the activity of RNAase from bovine pancreas, and it reduces the protein - nucleic acid interactions thereby producing greater fluorescence enhancement of ethidium bromide by the nucleic acids in the homogenate.

Since the estimates of the DNA content of the intestinal mucosa obtained by the method of Prasad et al (1972) were low and since the method of Karsten and Wollenberger (1972) gave a 25% increase in the estimated DNA content the latter method was adapted for use in this work.

Principle of the assay

The tissue is homogenized in the presence of a protease which inactivates endogenous nuclease activity. A sample of the homogenate, containing 5 µg or less DNA, is incubated with a buffer in the

presence of the protease. During this incubation the protease reduces the protein - nucleic acid interactions and maximum fluorescence enhancement is obtained subsequently upon addition of ethidium bromide. The sample is then incubated in the presence of RNAase and the fluorescence enhancement due to DNA alone is obtained. The fluorescence enhancement due to RNA is obtained from the difference between the two readings.

Preparation of an intestinal mucosa homogenate

The mucosal homogenate was prepared as described in Chapter 4 and samples were frozen at -40°C prior to estimation of the DNA content. Since the mucosal homogenate was also required for the estimation of peptide hydrolase activities, the protease was omitted from the homogenate at this stage since it inactivates peptide hydrolase activities (results not presented).

DNA estimation - Method finally adopted

The following solutions were prepared just before use:

Phosphate buffered saline	0.1 g CaCl_2 , 0.2 g KCl , 0.2 g KH_2PO_4 , 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 8.0 g NaCl , 1.15 g Na_2HPO_4 dissolved in 1 litre of distilled water and the pH was adjusted to 7.5 with NaOH (2mol/l) just before the final volume was reached. (All salts were AnalaR chemicals from B.D.H. Chemicals Ltd)
Ethidium bromide	2.5 g ethidium bromide (Sigma London Chemical Co.) in 200 ml phosphate buffered saline.

DNA and RNA

As described previously.

'Pronase'

10 mg 'Pronase' (Pronase P, Type VI, bacterial, purified, Sigma London Chemical Co.) in 100 ml phosphate buffered saline.

RNAase

As described previously.

The homogenate was thawed for 15 minutes at room temperature and then mixed well with a vortex mixer. A portion (0.1 ml) was diluted in the range 1:80 to 1:640 by serial dilution on ice with an automatic pipette as described previously. Tubes were prepared containing 0.2 ml sample, 0.8 ml 'Pronase' solution and 1 ml phosphate buffered saline. The quantities used by Karsten and Wollenberger (1972) have been doubled to give a convenient volume for fluorescence measurement. The samples were incubated for 20 minutes at 37°C in a water bath. After removal from the water bath 2 ml of ethidium bromide solution was added to each of the tubes which were then mixed and the fluorescence was read in a Perkin-Elmer 203 Fluorescence Spectrophotometer at an excitation wavelength of 360 nm and an emission wavelength of 580 nm. Zero emission intensity was set with a blank containing 1.2 ml buffer, 0.8 ml 'Pronase' and 2 ml ethidium bromide and the emission intensity was calibrated at 100% with a sample containing 10 µg DNA.

Stable readings were obtained without the use of surface fluorometry as recommended by Karsten and Wollenberger (1972).

RNAase (0.04 ml) was then added to the set of sample tubes which were mixed and incubated at 37°C in a water bath for 1 hour.

Karsten and Wollenberger (1972) used an incubation time of 20 minutes at 37°C and a lower concentration of RNAase (50 $\mu\text{g/ml}$). Under these conditions very little RNAase activity was observed and this was probably due to the different RNAase preparation used. The concentration used previously was therefore used in the assay. After removal of the samples from the water bath 2 ml of ethidium bromide was added and the fluorescence was read as above.

Calibration curves

A calibration curve in the range 10 - 50 $\mu\text{g DNA/ml}$ was prepared for each set of estimations. The curve was linear over the whole range (Fig. 5.2) and the DNA concentration of the test samples was calculated from the first order regression line of the calibration curve.

During investigation of the assay a calibration curve for RNA in the range 10 - 60 $\mu\text{g RNA/ml}$ was also prepared. However, since RNA was not estimated in the mucosal homogenates only the 60 $\mu\text{g RNA/ml}$ sample was routinely used as an index of the activity of the RNAase.

DNA stability

Karsten and Wollenberger (1972) stated that stock solutions of DNA may be stored at 4°C for 3 months without a decrease in fluorescence. However, as shown in Fig. 5.3, the fluorescence of a stock solution of DNA had decreased by 16% after storage at 4°C for 5 weeks and by 37% after storage at -40°C for 5 weeks (a stock solution of 50 $\mu\text{g/ml}$ DNA was divided into 1 ml portions; half were stored at 4°C and half at -40°C . The DNA content was assayed at

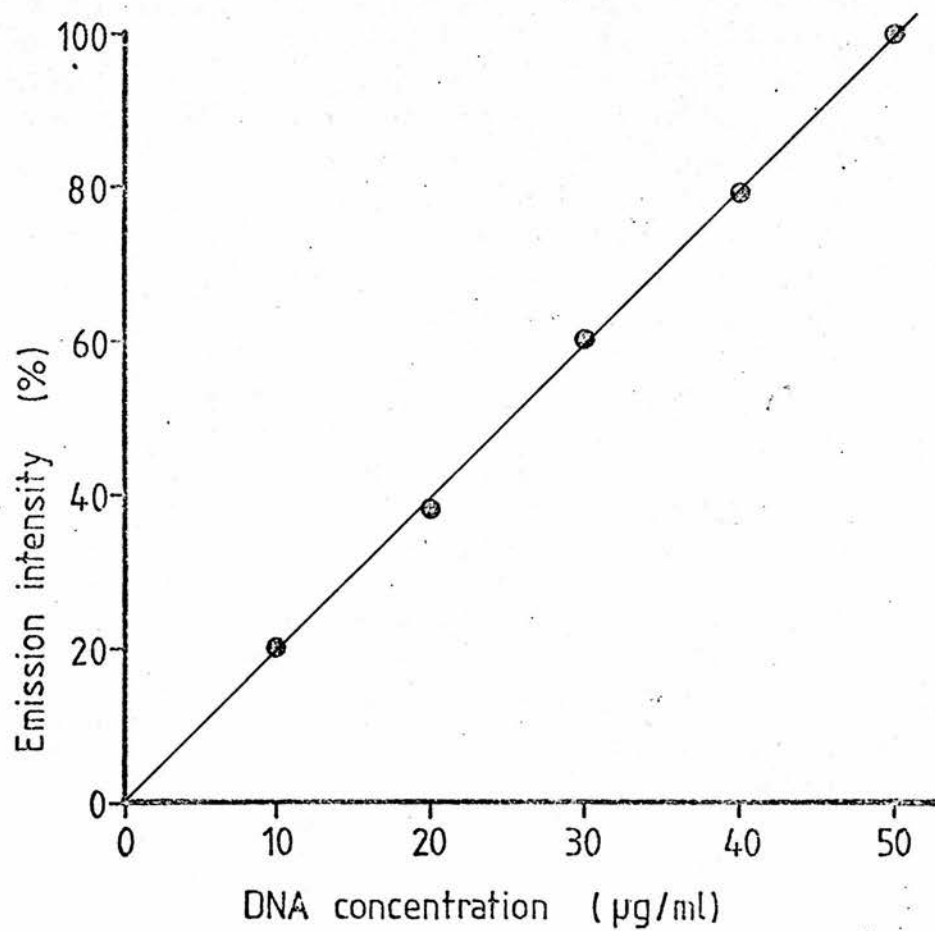


Fig. 5.2 Calibration curve for the estimation of DNA with ethidium bromide by the method of Karsten and Wollenberger (1972). Each point is the mean of 2 estimations.

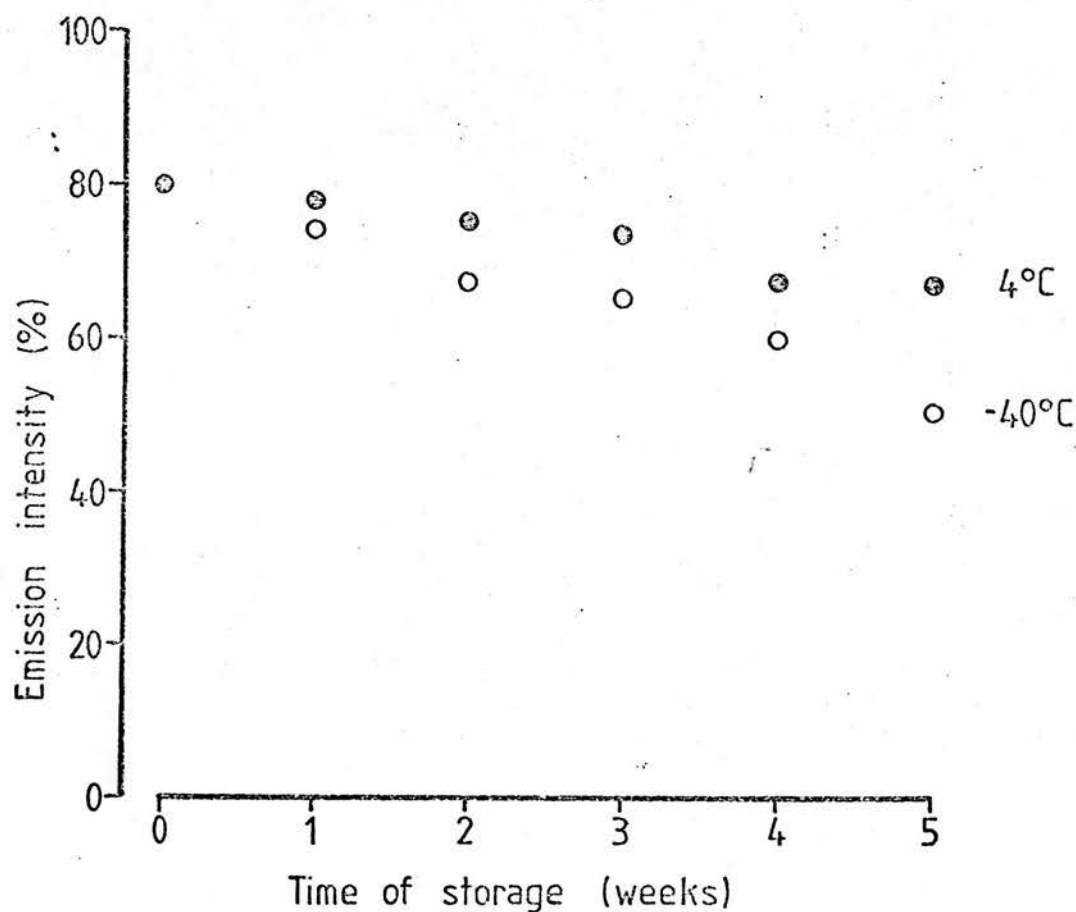


Fig. 5.3 Fluorescence enhancement of ethidium bromide by DNA estimated after storage of the DNA solution at 4°C (●) or -40°C (○) for various times. The fluorimeter was calibrated with a quinine sulphate fluorescence standard. Each point is the mean of 3 estimations.

1 week intervals and the fluorimeter was calibrated with a quinine sulphate fluorescence standard of 10 mg/l of 0.1 mol/l HCl).

A stock solution of DNA was therefore prepared freshly for each set of estimations.

Estimation of DNA in a mucosal homogenate

Estimations were made in duplicate at each of two dilutions unless otherwise stated. Initial estimations on 4 rats gave a mean mucosal DNA content of 29.4 ± 2.3 mg DNA/intestine and this compares well with estimates reported elsewhere (see Table 5.1).

Table 5.1 Values quoted in the literature for the DNA content of the mucosa from the rat small intestine

Authors	Weight of rat (g)	Mucosal DNA content (mg)
Altman and Enesco (1967)	100	26.7
"	300	47.7
Gardner and Plumb (1979)	180	26.5
Gardner and Plumb (1981)	180	32.5
McManus and Isselbacher (1970)	200	40.4
Steiner et al (1968)	370	22.2
Williamson and Malt (1980)	210	1.7 mg/5 cm

The estimates were reproducible; the coefficient of variation of an estimate was 2.7% (n = 10).

DNA and RNA recoveries

Portions (0.2 ml) of solutions of DNA in the range 10 - 40 μ g/ml

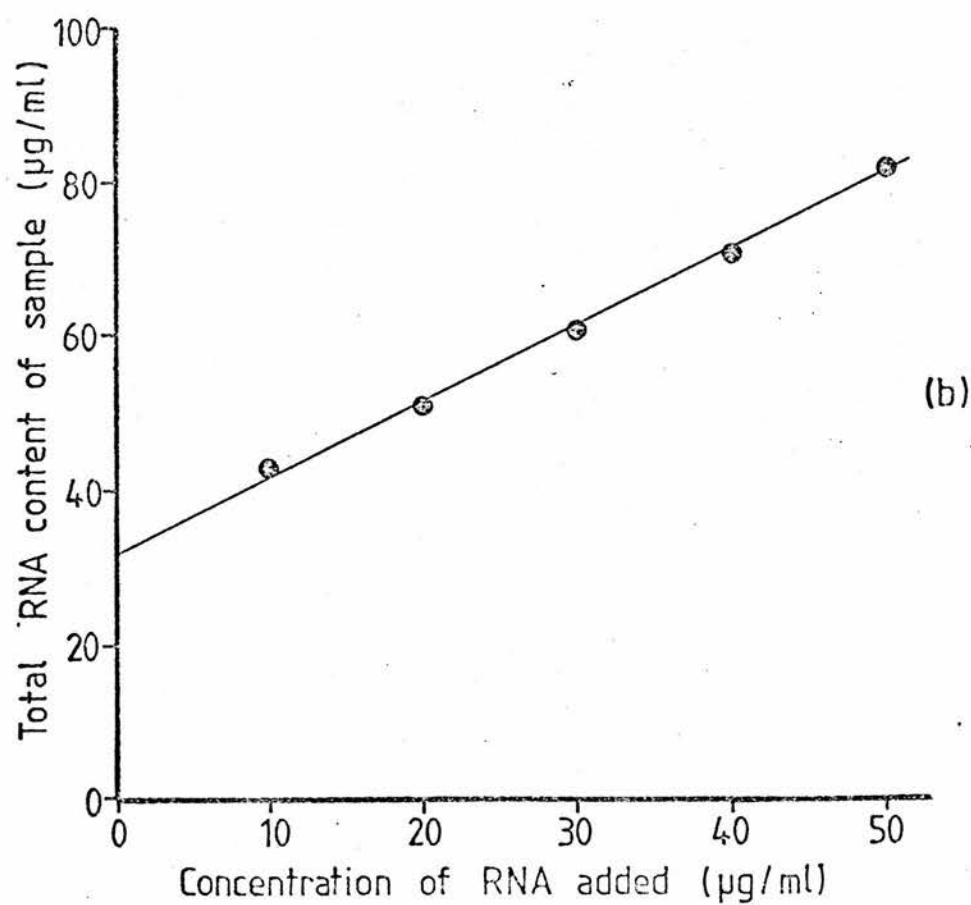
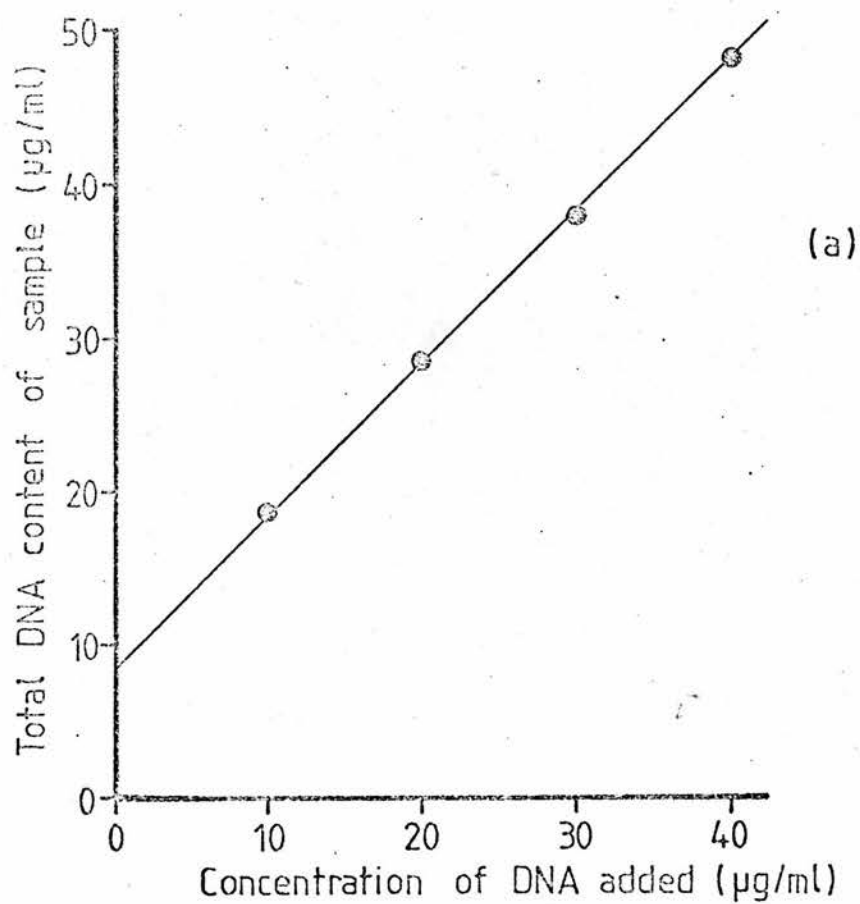
were added to 0.2 ml portions of a suitable dilution of a mucosal homogenate. The DNA content of the test samples was then estimated as described except that only 0.8 ml of phosphate buffered saline was used. Fig. 5.4a shows the DNA content of the test samples plotted against the amount of DNA added.

The recovery was very good; the slope of the regression line was 1.01 ± 0.02 (\pm standard error of the slope). The calculated intercept on the ordinate gives the DNA content of the mucosal homogenate (26.1 mg/intestine) which compares well with the estimated DNA content of the sample (25.7 mg/intestine).

The results for RNA recoveries are shown in Fig. 5.4b. Similarly the recovery was good; the slope of the regression line was 0.98 ± 0.03 (\pm standard error of the slope). The RNA content of the mucosal homogenate calculated from the intercept on the ordinate (102.4 mg/intestine) compares well with the estimated mucosal RNA content (98.7 mg/intestine).

Thus the recoveries were $101 \pm 2\%$ and $98 \pm 3\%$ for DNA and RNA respectively.

Fig. 5.4 The recovery of RNA and DNA added to a suitable dilution of a mucosal homogenate. The total DNA (a) or RNA (b) content of the sample is plotted against the amount added. Each point is the mean of 2 estimations.



DISCUSSION

The method of Karsten and Wollenberger (1972) with the modifications described in this chapter proved to be a suitable method for the estimation of the DNA content of a mucosal homogenate prepared from the small intestine of the rat. The results were reproducible and the recovery of added DNA was very good.

The introduction of a protease by Karsten and Wollenberger (1972) was beneficial to the measurement of DNA in an homogenate. Two further modifications have been introduced:

- (a) A fresh preparation of DNA is used for the calibration curve since a solution cannot be stored without loss of DNA.
- (b) The concentration of RNAase used has been increased and the incubation time at 37°C has been extended to 1 hour; this is probably a reflection on the activity of the enzyme obtained from a different source.

One problem associated with the assay was the variability in the activity of the RNAase. This variation was observed between different batches of RNAase and the activity of a batch of RNAase decreased with the time of storage. It was therefore essential to include the RNA standard solution in the assay in order to ensure that RNA digestion was complete after incubation for 1 hour with RNAase.

The action of both endogenous and exogenous nuclease activities presents a major problem during the measurement of the nucleic acid content of a mucosal homogenate. This activity was minimized by the inclusion of a protease in the assay and also by the precaution of wearing P.V.C. gloves during handling of the intestine. If the DNA

content of a mucosal homogenate could not be estimated immediately, as in the experiments described in Chapter 6, the homogenate was stored frozen at -40°C for up to 5 hours. When samples were stored at 4°C for 5 hours the mucosal DNA content of intestines removed from anaesthetized animals was decreased by about 25% (see Figs. 6.5 and 6.8) and it is possible that this decrease was due to nuclease activity even at 4°C .

CHAPTER 6

Release of dipeptide hydrolase activities from rat small intestine perfused in vitro and in vivo

Introduction

Although it has been known since the turn of the century that animals fed on protein that has been completely hydrolysed to amino acids can maintain a positive nitrogen balance (Henderson and Dean, 1903), the form in which protein is normally absorbed from the intestinal lumen is not known and is the subject of much debate. Originally it was assumed that proteins were absorbed as polypeptides (see review by Matthews, 1975). However, in 1901 Cohnheim demonstrated that the fluid in the intestinal lumen (the 'succus entericus') contained peptide hydrolase activity ('erepsin') (Cohnheim, 1901, cited by Matthews, 1975). From this observation developed the belief that all protein entering the intestine was completely hydrolysed to amino acid prior to absorption (e.g. Evans, 1949; Verzar and McDougall, 1936). This belief was held so rigorously that a number of studies which demonstrated that peptides were absorbed more rapidly than amino acids in the human were ignored (e.g. Heath and Fullerton, 1935; Messerli, 1913). It was not until 1959 when Newey and Smyth showed that the dipeptide glycylglycine was taken up in intact form from the intestinal lumen that this theory was seriously questioned. It is now known that there is a significant hydrolysis of small peptides on the brush border and in the cytoplasm of intestinal mucosal cells (Smyth, 1972). However, the relative importance of transport of amino acids and peptides is unknown (Crampton et al, 1971); nor is it known whether or not a significant

amount of these transported peptides enter the blood stream without hydrolysis (Matthews, 1975). These observations have attracted attention to the existence of peptide hydrolase enzymes in the small intestinal mucosa.

Peptide hydrolase enzymes are present in the intestinal lumen as well as on the brush border and in the cytoplasm of the intestinal mucosal cells. The physiological significance of the luminal activity, thought to originate predominantly from the mucosal cytoplasm (Silk and Kim, 1975), is not known but some workers argue that it is insufficient to account for the total hydrolysis to amino acids of a protein meal (Cajori, 1933; Fisher, 1954). On the other hand, it has been suggested that the release of dipeptidase enzymes from the small intestine in vivo may be a physiological process and that it responds to various specific stimuli such as the hormones gastrin, caerulein and glucagon (Eloy et al, 1978) and cholecystokinin - pancreozymin (Götze et al, 1972).

Alternatively, since some intestinal preparations in vitro release considerably greater amounts of peptidases into the incubation medium, other workers have suggested that the release is simply an artifact associated with a dying tissue (Lindberg, 1972; Silk and Kim, 1976).

If peptide hydrolase enzymes are secreted into the intestinal lumen, then this might be an important site of terminal digestion of protein. However, if the release is abnormal and is associated only with intestinal preparations in vitro then the results of studies of the physiological relationship between peptide transport and hydrolysis made with such preparations may not be valid; a point

stressed by Silk and Kim (1976).

As discussed in Chapters 4 and 5 the procedures chosen in this work for the estimation of both mucosal DNA content and cytoplasmic peptide hydrolase activities involve estimating these in the mucosa from the entire jejunum plus ileum in order to avoid the use of an alternative reference basis. Although Gardner and Heading (1979) estimated peptide hydrolase activities on 1 cm segments of jejunum and ileum these activities need not be representative of activities in the whole small intestine since these activities are known to vary in different regions of the small intestine (Josefsson and Lindberg, 1965; Robinson and Shaw, 1960). Thus the whole small intestine was chosen as a reference system since this also overcomes the need to use a reference system such as protein, unit length or weight of intestine, all of which are variable with respect to the whole small intestine, and which are themselves subject to variation during, e.g., 5-fluorouracil treatment or food restriction. It was therefore important to determine whether mucosal DNA contents and cytoplasmic peptide hydrolase activities could be determined on an intestine which had already been used for a perfusion experiment or whether additional unperfused intestines were necessary for these estimations. If the former were possible then water absorption rates, mucosal DNA contents and cytoplasmic peptide hydrolase activities could all be estimated on the same intestine. Not only would this reduce the number of animals required, it would also provide information on the inter-relationship of the three parameters. In particular, in Chapter 8, the mucosal DNA content is used as an index of the number of mucosal cells in an attempt to determine whether the changes in the intestinal water absorption rates following 5-fluorouracil injection are caused by (a) a change

in the number of absorbing cells, (b) a change in the absorptive capacity of individual absorbing cells or (c) a combination of these two. Obviously it would be preferable in such a study to be able to estimate absorption rates and mucosal DNA contents on the same intestine.

Thus, in view of the previous observations that peptide hydro-
lase activities are released from preparations in vitro, a study was
designed to determine whether intestines, after perfusion, could be
used for the estimation of mucosal DNA contents and cytoplasmic
peptide hydrolase activities. This study was extended to intestines
perfused in vivo in an attempt to give information on the physio-
logical significance of the release of peptide hydrolase activities
from the small intestine. The technique of Fisher and Gardner (1974)
described in Chapter 3 has the advantage that intestines can be
perfused on the same apparatus either in vitro or in vivo and thus,
unlike any of the previous studies of enzyme release (Götze et al,
1972; Josefsson and Sjöström, 1966; Silk and Kim, 1975 and 1976;
Silk et al, 1976) a direct comparison can be made between the
enzyme release in vitro and in vivo. When intestines are perfused
in vitro by the technique of Fisher and Gardner (1974) specific care
is taken to ensure that the intestine is never deprived of a supply
of oxygen; the rat is maintained under ether anaesthesia until
establishment of luminal perfusion and removal of the intestine from
the rat have been completed (see Chapter 3), a precaution recommended
by Fisher and Parsons (1949) and Fisher and Gardner (1974). Since
failure to abide by these precautions results in a 50% decrease in
the ^{and} glucose_{and} water absorption rates by the rat small intestine
perfused in vitro (Gardner, 1978), a second preparation in vitro in

which the intestine for perfusion is rapidly removed from a freshly killed rat has been included in the study of enzyme release. Thus three intestinal preparations were used with a view to determining whether the release of peptidase enzymes from perfused intestines differs in vitro and in vivo and also whether the release from a preparation in vitro depends to an appreciable extent on whether the intestine is deprived of oxygen at any point during preparation for perfusion.

Peptide hydrolase activities were estimated in the mucosal cytoplasm from unperfused and in perfused intestines and in the luminal effluents and serosal secretions from perfused intestines. Since the luminal activity might originate from cells sloughed off the intestinal mucosa during perfusion, the mucosal DNA content has been estimated in unperfused and in perfused intestines in order to determine the extent of cell sloughing during perfusion.

A preliminary study was designed to test the suitability of the peptidase assay and the DNA assay chosen for use in this work and also to give an indication of the extent of the release of enzyme activities. This study revealed a number of problems, discussed below, which lead to the introduction of a different method for the estimation of peptide hydrolase activities. The results of the preliminary study are therefore included in this chapter since they are pertinent to the choice of peptide hydrolase assay used in this work and since the information gained from this study was then used as the basis of the study of the physiological significance of the release of peptide hydrolase activities from the perfused small intestine.

The principal results from this investigation have been presented to the European Intestinal Transport Group (Plumb and Gardner, 1979) and have been published in full (Gardner and Plumb, 1979).

Part I: Preliminary observations

METHODS

Animals

Female rats, weighing 160 - 200 g, were used throughout this study.

Intestinal perfusion

Perfusion in vitro: Whole jejunum plus ileum from the ligament of Treitz to the ileo-caecal valve was perfused in vitro by the method of Fisher and Gardner (1974), described in Chapter 3, for 0, 20, 40 or 60 minutes. Intestines perfused for 0 minutes were removed from the rat immediately after the lumen had been washed through with the oxygenated bicarbonate-saline (see p. 27). The luminal effluent and serosal secretion were collected over the entire perfusion period at room temperature. Experiments were performed on 4 separate days: on each day 4 rats were used, one for each duration of perfusion. The order of these experiments on each day was varied according to a pseudo-random Latin Square design.

The precautions of Fisher and Gardner (1974) to avoid temporary anoxia were strictly observed: if any animal died then the experiment was abandoned.

Perfusion in vitro of intestines from freshly killed rats:

Whole jejunum plus ileum from the ligament of Treitz to the ileo-caecal valve was perfused by the method of Fisher and Gardner (1974) except that the ether-anaesthetized rats were killed by stunning and cervical dislocation immediately before the intestine was removed in

the usual way for perfusion. Intestines were perfused for 0 or 60 minutes and those perfused 0 minutes were removed from the rat immediately after the lumen had been washed through with oxygenated bicarbonate-saline.

Perfusion in vivo: Animals were anaesthetized 1 hour before use with urethane (1 ml of 0.8 mg/ml solution injected subcutaneously) while under temporary light ether anaesthesia. The technique and apparatus of Fisher and Gardner (1974) were used except that the small intestine (jejunum plus ileum) was left in situ partly outside the abdominal cavity with the vasculature intact. The intestine was covered with moist cotton wool and a polyethylene sheet and was maintained at approximately 38°C by an overhead lamp. As with the experiments in vitro the intraluminal distension pressure was about 30 cm water and the perfusion rate was about 3.5 ml/minute. The luminal effluent was collected at room temperature over the entire 60 minute perfusion period; there was no serosal secretion from this preparation. Intestines perfused for 0 minutes were removed from the rat immediately after the lumen had been rinsed through with oxygenated bicarbonate-saline.

Peptide hydrolase assay

Hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu were estimated by the method of Gardner et al (1978), described in Chapter 4, in the supernatant of the mucosal homogenate of the whole jejunum plus ileum, in the serosal secretions and the luminal effluents. Supernatant samples were diluted within the range 1:320 to 1:1280 and the serosal secretions and luminal effluents were used undiluted. All assays were carried out in

duplicate and enzyme activities were expressed in terms of total enzyme units in the mucosa, luminal effluent or serosal secretion where 1 unit is defined as the amount catalysing the hydrolysis of 1 μ mol of substrate/minute under the conditions of temperature and pH given in Chapter 4.

Estimations were carried out on the same day as, but not until completion of, all the perfusion experiments. The mucosal homogenates were prepared immediately after perfusion and all samples were stored at 4°C until the assays were carried out on them.

Mucosal DNA estimation

DNA in the mucosal homogenate of the whole small intestine was estimated by the method of Le Pecq and Paoletti (1966) as modified by Karsten and Wollenberger (1972) with further modifications as described in Chapter 5. Samples were estimated in duplicate at each of three dilutions, 1:80, 1:160 and 1:320, and the assays were carried out after completion of the perfusion experiments. The homogenates were stored at 4°C until the assay was carried out on them.

RESULTS

Peptide hydrolase activities in the luminal effluents and serosal secretions

Fig. 6.1 shows the dipeptidase activities determined in the luminal effluents and secretions on to the serosal surface of the intestine pooled over the perfusion period of 1 hour. Activities estimated in the luminal effluent from perfused intestines that had been rapidly removed from freshly killed rats were two to four times greater than those estimated in the luminal effluents either from intestines perfused in vitro and removed from anaesthetized rats or from intestine perfused in vivo. Similarly activities estimated in the serosal secretions were greater by a factor of 2 when intestines, perfused in vitro, had been removed from freshly killed rats.

The release of activities into the luminal effluent appears to be a continuous process throughout the perfusion period (Fig. 6.2).

Activities estimated in the luminal effluents and serosal secretions were all very low and close to the lower limit of sensitivity of the assay except in the experiments on intestines from freshly killed rats.

Cytoplasmic peptide hydrolase activities of intestines before and after perfusion

Fig. 6.3 shows the hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu in the mucosal cytoplasm from whole jejunum plus ileum of unperfused intestines and of intestines after perfusion for 1 hour.

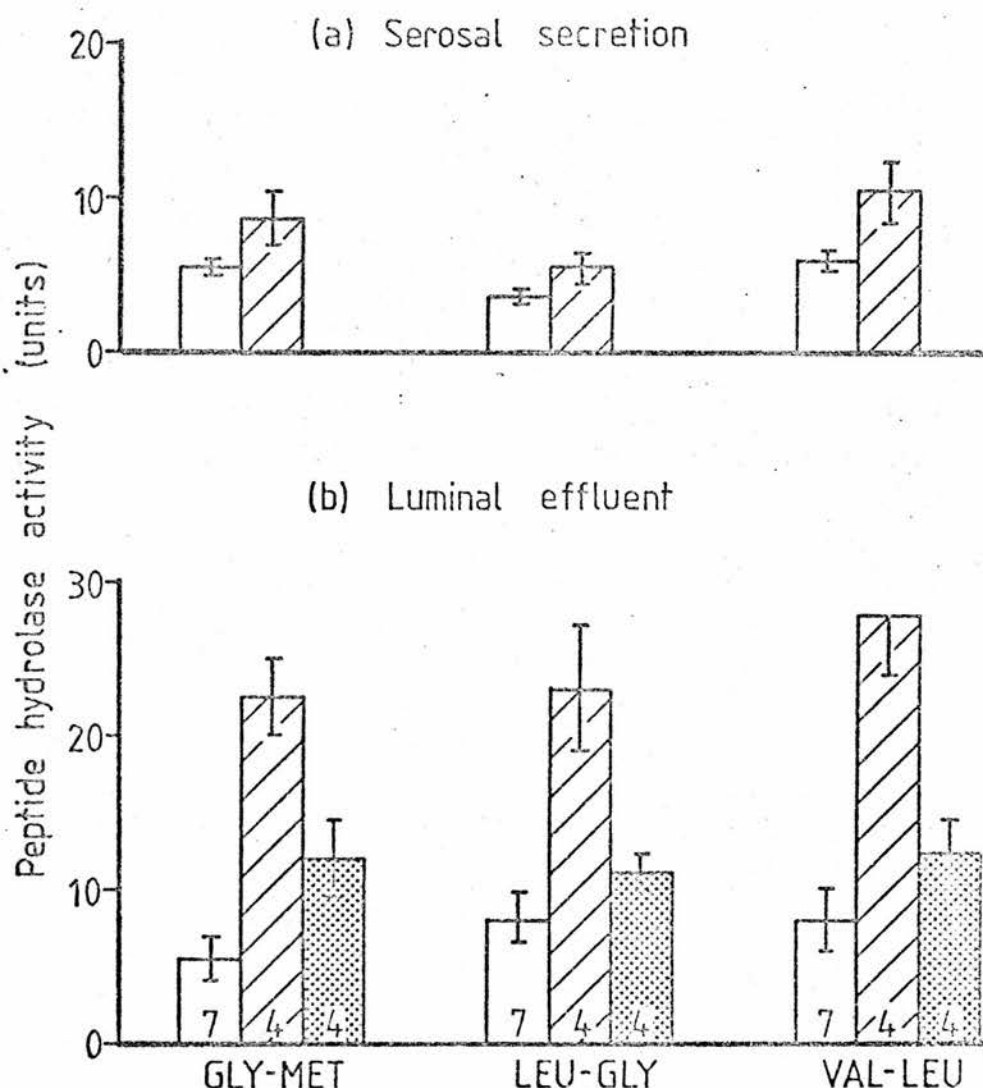


Fig. 6.1 Peptide hydrolase activities recovered in (a) secretion on to the serosal surface of the intestine, and (b) luminal effluent during perfusion for 1 hour *in vitro* of intestines prepared from ether-anaesthetized rats (open bars), *in vitro* of intestines prepared from freshly killed rats (hatched bars) and *in vivo* (stippled bars). Values are means \pm S.E.M. and the number of intestines is shown within the bars in (b). Results are shown for the dipeptidase activities against three substrates, Gly-L-Met, L-Leu-Gly and L-Val-L-Leu.

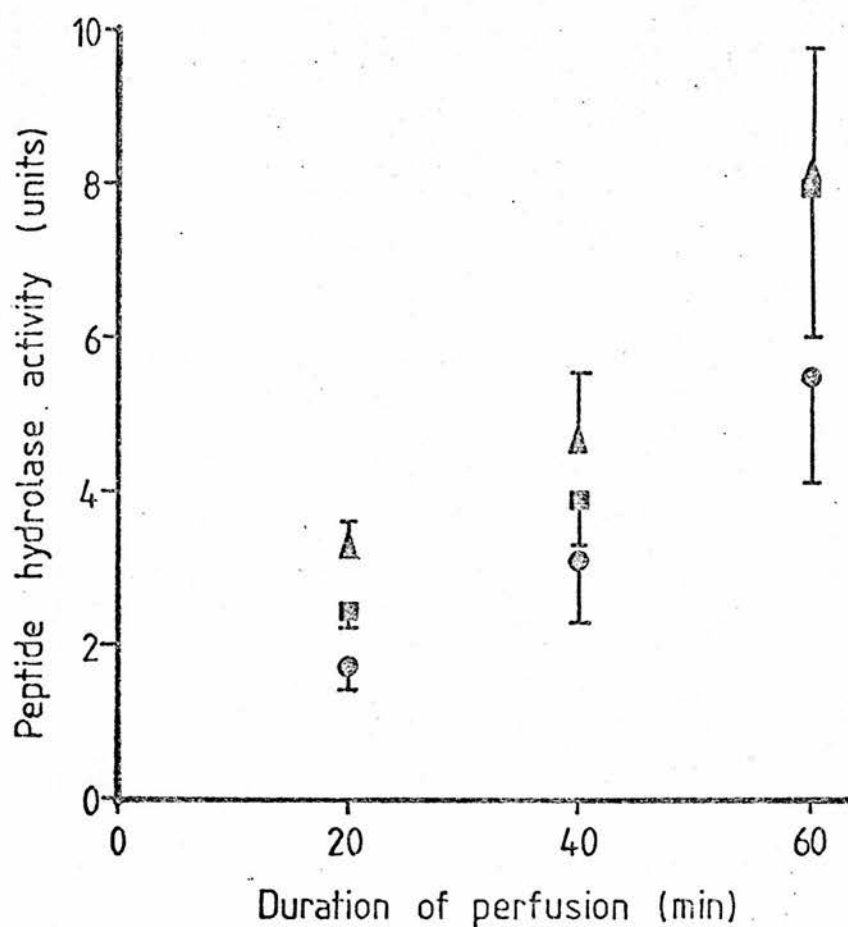
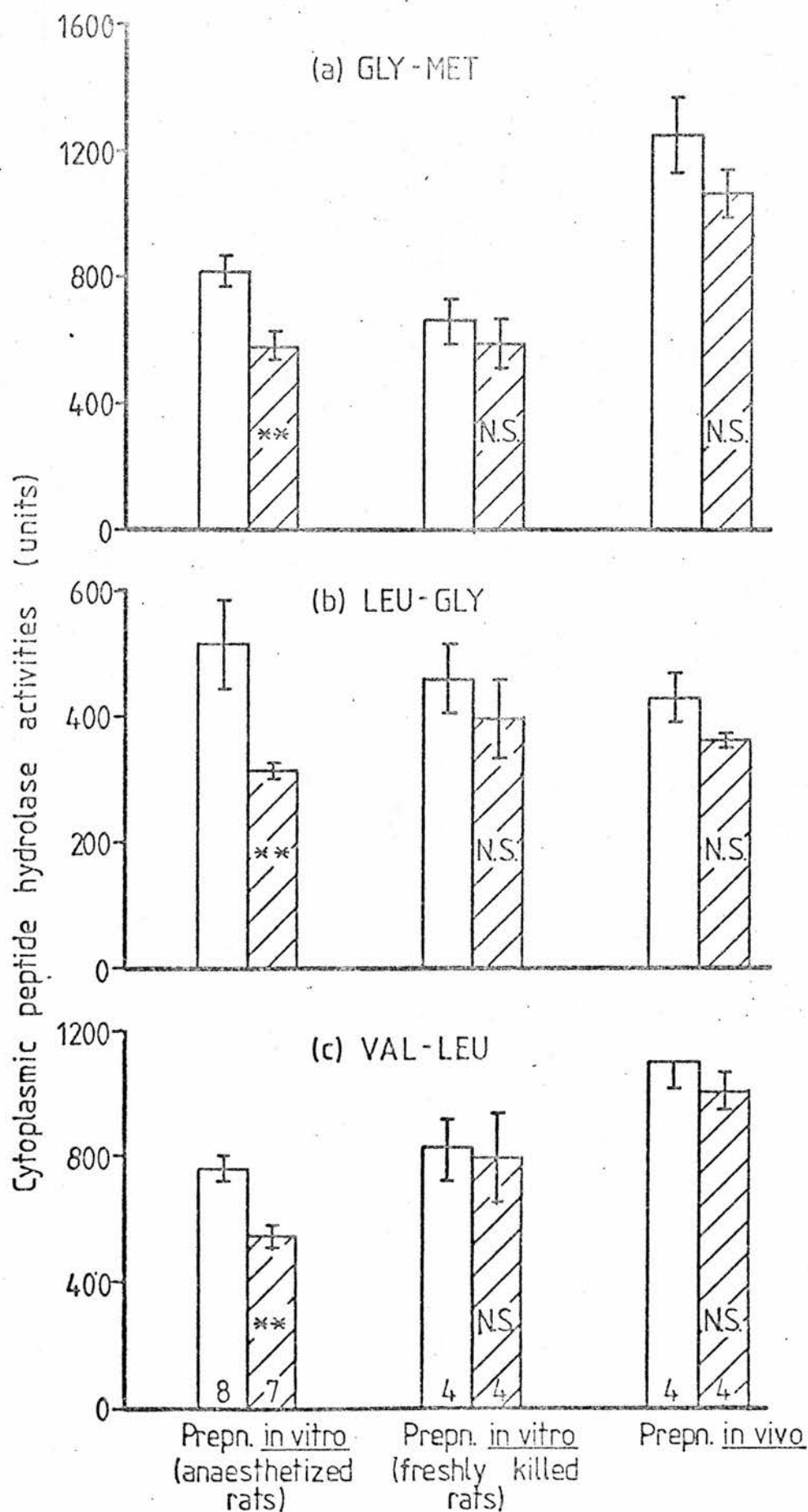


Fig. 6.2 Peptide hydrolase activities in luminal effluent from intestines prepared from ether-anaesthetized rats and perfused in vitro for 20, 40 and 60 minutes. Values are the mean \pm S.E.M. of observations on 4, 4 and 7 intestines respectively. Substrates were (●) Gly-L-Met, (▲) L-Leu-Gly, and (■) L-Val-L-Leu.

Fig. 6.3 Cytoplasmic peptide hydrolase activities in the mucosa of unperfused small intestines (open bars) and of small intestines perfused for 1 hour in vitro or in vivo (hatched bars). Intestines for perfusion in vitro were prepared both from ether-anaesthetized rats and from freshly killed rats. Substrates were (a) Gly-L-Met, (b) L-Leu-Gly, and (c) L-Val-L-Leu. Values are the mean \pm S.E.M. and the number of animals is shown within the bars in (c). The significance levels of the differences between the values in unperfused intestines are indicated thus: **P < 0.01, N.S. P > 0.1.



In all cases cytoplasmic peptide hydrolase activities were decreased after perfusion but the decrease was not always significant. Activities against Gly-L-Met and L-Val-L-Leu were slightly, but not significantly, less in unperfused intestines removed from freshly killed rats than those of intestines removed from ether-anaesthetized rats; in contrast, activities against Gly-L-Met and L-Val-L-Leu were significantly greater ($P < 0.01$) in unperfused intestines removed from urethane anaesthetized animals.

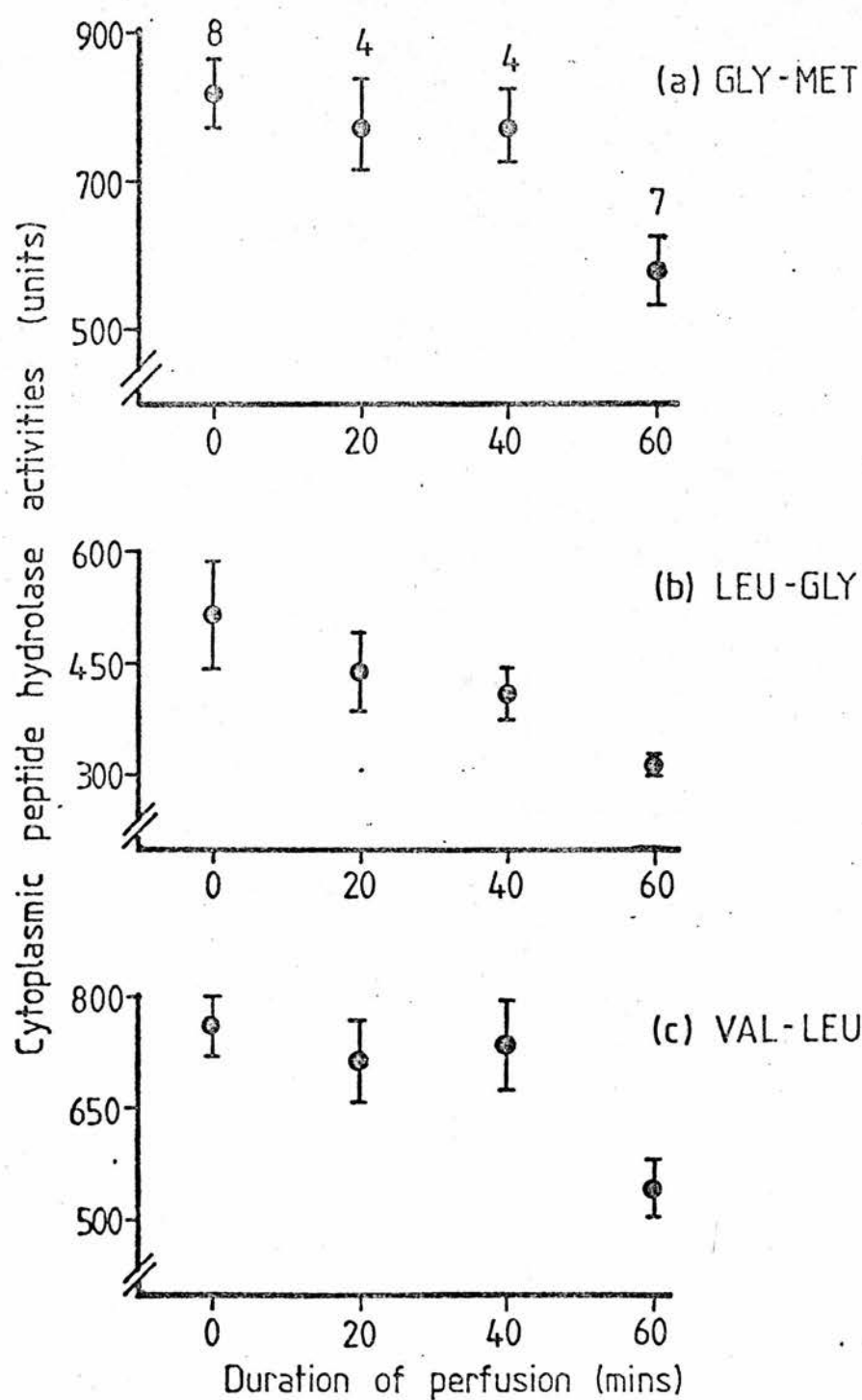
The loss of cytoplasmic peptide hydrolase activities from intestines perfused in vitro appears to be a continuous process throughout the period of perfusion for activities against L-Leu-Gly but appears to increase after 40 minutes of perfusion for activities against Gly-L-Met and L-Val-L-Leu (Fig. 6.4). However, in most instances, activities estimated in the supernatant fraction of the mucosal cytoplasm were inaccurate due to low reproducibility of estimates.

Mucosal DNA content of unperfused and of perfused intestines

Fig. 6.5 shows the mucosal DNA content of unperfused intestines and of intestines after perfusion for 1 hour. The mucosal DNA content (relative to that in unperfused intestines) was less after perfusion in vitro of intestines removed from ether-anaesthetized rats or from freshly killed rats although the decrease was not significant ($P > 0.1$). In contrast, perfusion in vivo for 1 hour had no effect on the mucosal DNA content (Fig. 6.5).

However, it is of note that these estimates of the mucosal DNA content of intestines removed from ether-anaesthetized rats were about 20% lower than those estimated previously (see Chapter 5).

Fig. 6.4 Cytoplasmic peptide hydrolase activities in mucosal supernatant after perfusion in vitro of intestines, prepared from ether-anaesthetized rats, for 20, 40 or 60 minutes. Substrates were (a) Gly-L-Met, (b) L-Leu-Gly, and L-Val-L-Leu. Values are means \pm S.E.M. and the number of intestines is shown above the bars in (a).



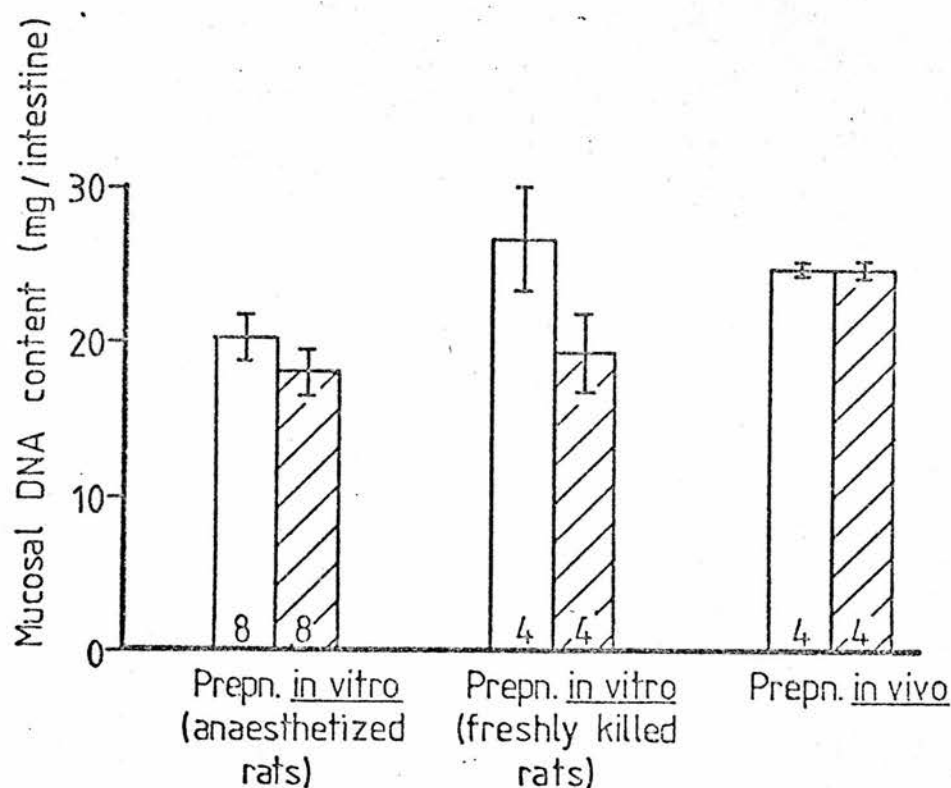


Fig. 6.5 DNA content of whole small intestinal mucosa of unperfused intestines (open bars) and of intestines after perfusion for 1 hour in vitro or in vivo (hatched bars). Intestines for perfusion in vitro were prepared both from ether-anaesthetized rats and from freshly killed rats. Values are the mean \pm S.E.M. and the number of intestines is shown within the bars.

Total recovery of peptide hydrolase activities after perfusion

The total peptidase activity recovered in the luminal effluent serosal secretion and mucosal cytoplasm after perfusion for 1 hour amounted to only 60.3% (mean of all experiments) of the activity in the mucosal cytoplasm of unperfused intestines.

DISCUSSION

The results of the preliminary study show that peptide hydrolase activities are released from the intestine during perfusion in vitro and in vivo. The amount of activity released into the luminal effluent and serosal secretion during perfusion in vitro appears to be influenced by the technique used during preparation of the intestine for perfusion; activities were two to three times greater when the intestine had been removed from a freshly killed animal than the activities found in the luminal effluent and serosal secretion from intestines removed from anaesthetized animals (Fig. 6.1). Also, there is a small loss of DNA from the mucosa during perfusion in vitro but not during perfusion in vivo (Fig. 6.5). Although all three peptide hydrolase activities estimated in the mucosal cytoplasm were decreased by perfusion in vitro or in vivo for 1 hour the decrease was only significant for activities against Gly-L-Met (Fig. 6.3).

However, the study also revealed a number of problems associated both with the peptide hydrolase and DNA assays and with the design of the study.

The peptide hydrolase assay

The problems associated with the use of this assay are described in detail in Chapter 4. It became apparent that the assay, as used by Gardner et al (1978), was not sufficiently sensitive to estimate reliably peptide hydrolase activities in the luminal effluent and serosal secretion. Furthermore, peptide hydrolase activities estimated at different dilutions of the mucosal cytoplasm did not agree and often duplicate samples at the same dilution differed.

The results of the preliminary study are therefore probably inaccurate and are not discussed in detail. However, it appears probable that the release of peptide hydrolase activity during perfusion is a gradual process throughout the period of perfusion rather than one which commences after a period of perfusion (Figs. 6.2 and 6.4). If this is so then it is not necessary to estimate the release after various durations of perfusion; a single estimate will be representative of the amount of activity released from the preparation during perfusion.

It was noted that the intestinal cytoplasmic peptide hydrolase activities varied between animals and that activities against Gly-L-Met and L-Val-L-Leu estimated in the intestines removed from urethane anaesthetized animals were significantly greater ($P > 0.01$) than those estimated in intestines removed from ether-anaesthetized animals. These differences were not observed subsequently (see Fig. 6.7) and were probably due to the inaccuracy of the method used to assay the activities.

The DNA assay

The mucosal DNA contents of intestines removed from ether-anaesthetized animals were consistently about 20% less than those estimated previously (Chapter 5). The reason for this discrepancy is not known but it is possible that the delay of up to 5 hours between preparation of the mucosal homogenate and estimation of the DNA content might have been responsible. Samples were stored at 4°C but it is possible that some nuclease activity remains at this temperature.

Stability of peptide hydrolase activities

Peptide hydrolase activities are known to be thermolabile

(Heizer et al., 1972) and a subsequent study of the effects of temperature on peptidase activities in the mucosal cytoplasm has confirmed this (see Chapter 4). Since the luminal effluents and serosal secretions were collected at room temperature over a period of 1 hour* and furthermore, since all samples were stored for up to 5 hours at 4°C prior to estimation of peptidase activities, it is probable that the activities estimated in these samples were much lower than the true activities due to the heat inactivation of the enzyme activities during the experiment. This could well explain the very low recovery (60%) of activities after perfusion.

* an experimental approach similar, in effect, to that used by Silk and Kim (1976)

Part II: The release of peptide hydrolase activities from the small intestine perfused in vitro and in vivo

The preliminary observations provided substantial information upon which was based the following study of the release of peptide hydrolase activities during perfusion in vitro and in vivo.

In order to overcome (a) the insensitivity of the peptide hydrolase assay and (b) thermal lability of the hydrolase enzymes the study was redesigned to allow estimation of peptide hydrolase activities in the luminal effluents and serosal secretions as soon as possible after completion of perfusion and a new assay was developed for the estimation of these activities.

METHODS

Intestinal perfusion

The three preparations described in the preliminary study were used and intestines were perfused for 0 and 60 minutes only.

Peptide hydrolase assays

Hydrolase activities against Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu were estimated by the modified method of Nicholson and Kim (1975), described in Chapter 4, in the supernatant of the mucosal homogenate of whole jejunum plus ileum, in the serosal secretion and in the luminal effluent. Samples were diluted on ice in the range 1:320 to 1:2560 for supernatants, 1:4 to 1:30 for secretions and 0 to 1:2 for luminal effluents. All assays were carried out in triplicate and enzyme activities were expressed in

terms of total enzyme units in the mucosa, luminal effluent or serosal secretion, where 1 unit is defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol of substrate/minute under the conditions described in Chapter 4.

Mucosal DNA estimation

No changes were made in the method described in Part I except that samples were stored at -40°C prior to assay the same day.

Experimental design

The modifications were concerned mainly with ensuring that all material for enzyme assay was processed as rapidly as possible.

Luminal effluents and serosal secretions were collected into glass measuring cylinders surrounded by ice. Ten minutes before the completion of a 60 minute perfusion experiment a mucosal homogenate was prepared from an unperfused intestine ($t = 0$). After preparation of a mucosal homogenate from the perfused intestine a portion of each homogenate was frozen at -40°C for subsequent estimation of DNA and a second portion was centrifuged immediately at 20 000 g at 4°C for 1 hour. During this hour peptide hydrolase activities were assayed in the luminal effluent and serosal secretion. Peptide hydrolase activities were assayed in the supernatant of the mucosal homogenates immediately upon completion of centrifugation.

This procedure was repeated for a second pair of intestines (perfused and unperfused) and upon completion the mucosal DNA contents of the homogenates from all four intestines were assayed.

RESULTS

Appearance of peptide hydrolase activities in luminal effluents and serosal secretions

Peptide hydrolase activities against Gly-L-Leu, L-Leu-Gly and L-Val-L-Leu were estimated in the luminal effluents and the secretions on to the serosal surface of the intestine pooled over the perfusion period of 1 hour.

The results are shown in Fig. 6.6 which also shows in parentheses these activities expressed as a percentage of the activities in the mucosal cytoplasm of the unperfused intestines. Significant amounts of dipeptidase activities were found in the luminal effluents during perfusion in vitro and in vivo. Only small amounts were found in the luminal effluents during perfusion in vivo or perfusion in vitro of intestines prepared from anaesthetized rats. In contrast, when intestines for perfusion in vitro were prepared from freshly killed rats (hatched bars in Fig. 6.6), the amounts of dipeptidase activity found in the luminal effluents were dramatically greater by factors of from 3.8 (against L-Leu-Gly) to 7.8 (against Gly-L-Met).

Similarly, small amounts of activity were found in the secretion on to the serosal surface of the intestine. A corresponding increase (approximately three-fold) was seen in the activities found in the serosal secretion from intestines prepared from freshly killed rats. However, the total activity found in the serosal secretions was invariably much less than that in the luminal effluents. When intestines from anaesthetized rats are perfused in vitro less than 1.9% of the initial cytoplasmic activity is recovered in the luminal effluent and less than 0.5% in the serosal secretion over 1 hour.

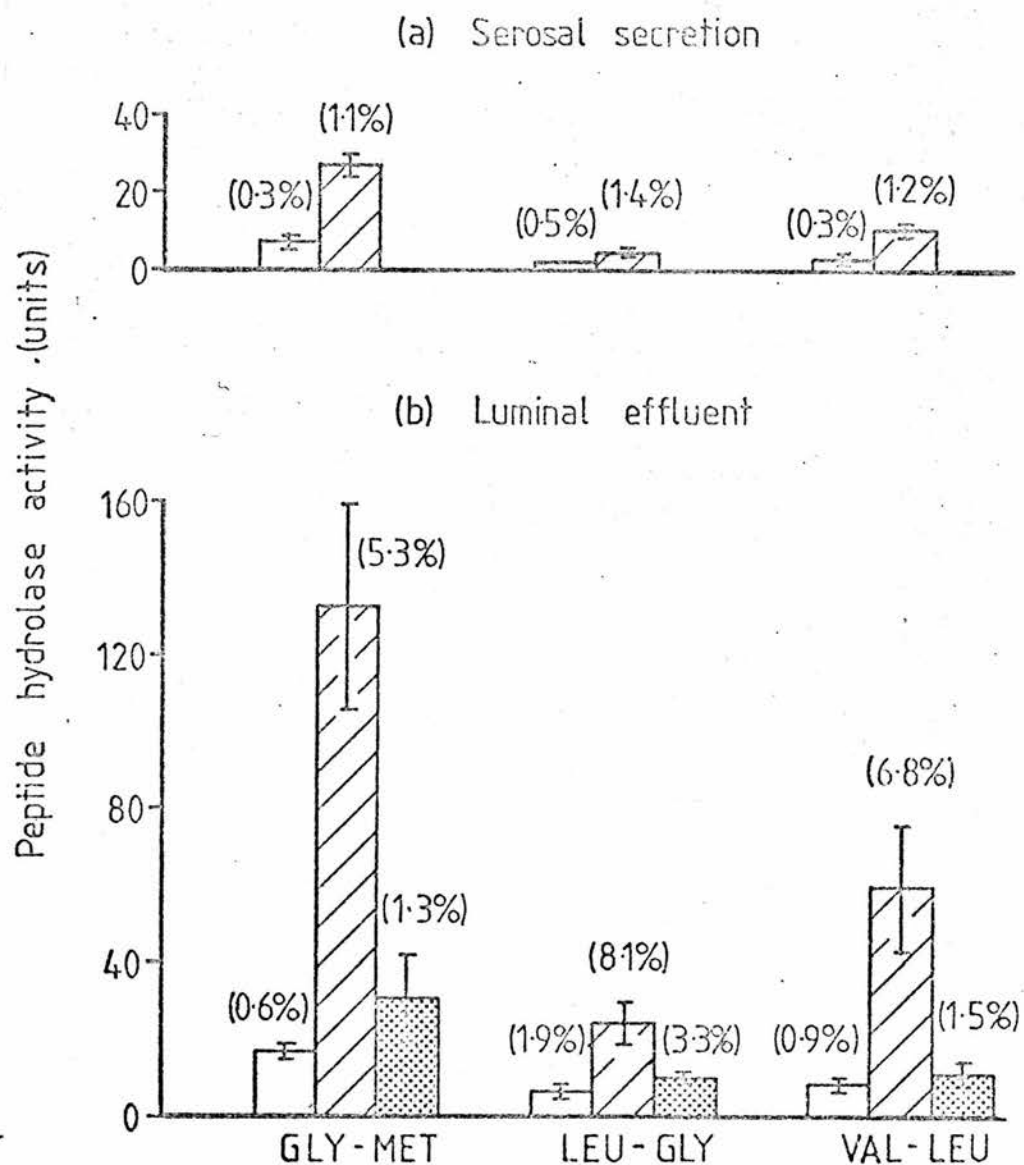


Fig. 6.6 Peptide hydrolase activities in (a) secretion on to the serosal surface of the intestine, and (b) luminal effluent during perfusion for 1 hour *in vitro* (10 intestines prepared from ether-anaesthetized rats) (open bars), *in vitro* (7 intestines prepared from freshly killed rats) (hatched bars) and *in vivo* (stippled bars, 4 rats). Values are means \pm S.E.M. Values in parentheses show mean values expressed as percentages of activities found in mucosal cytoplasm of unperfused intestines.

Cytoplasmic peptide hydrolase activities of unperfused and of perfused intestines

Fig. 6.7 shows the dipeptidase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu in the mucosal cytoplasm of unperfused intestines and of intestines after perfusion for 1 hour.

In all cases there was less cytoplasmic dipeptidase activity (8-32% less) after perfusion than in the unperfused intestines, although this difference was not always significant (see Fig. 6.7).

The peptidase activities in the unperfused, but saline-rinsed, intestines were slightly but not significantly ($P > 0.2$) lower in the killed animals and those anaesthetized by urethane (i.e. the controls for those perfused in vivo) than in the ether-anaesthetized ones.

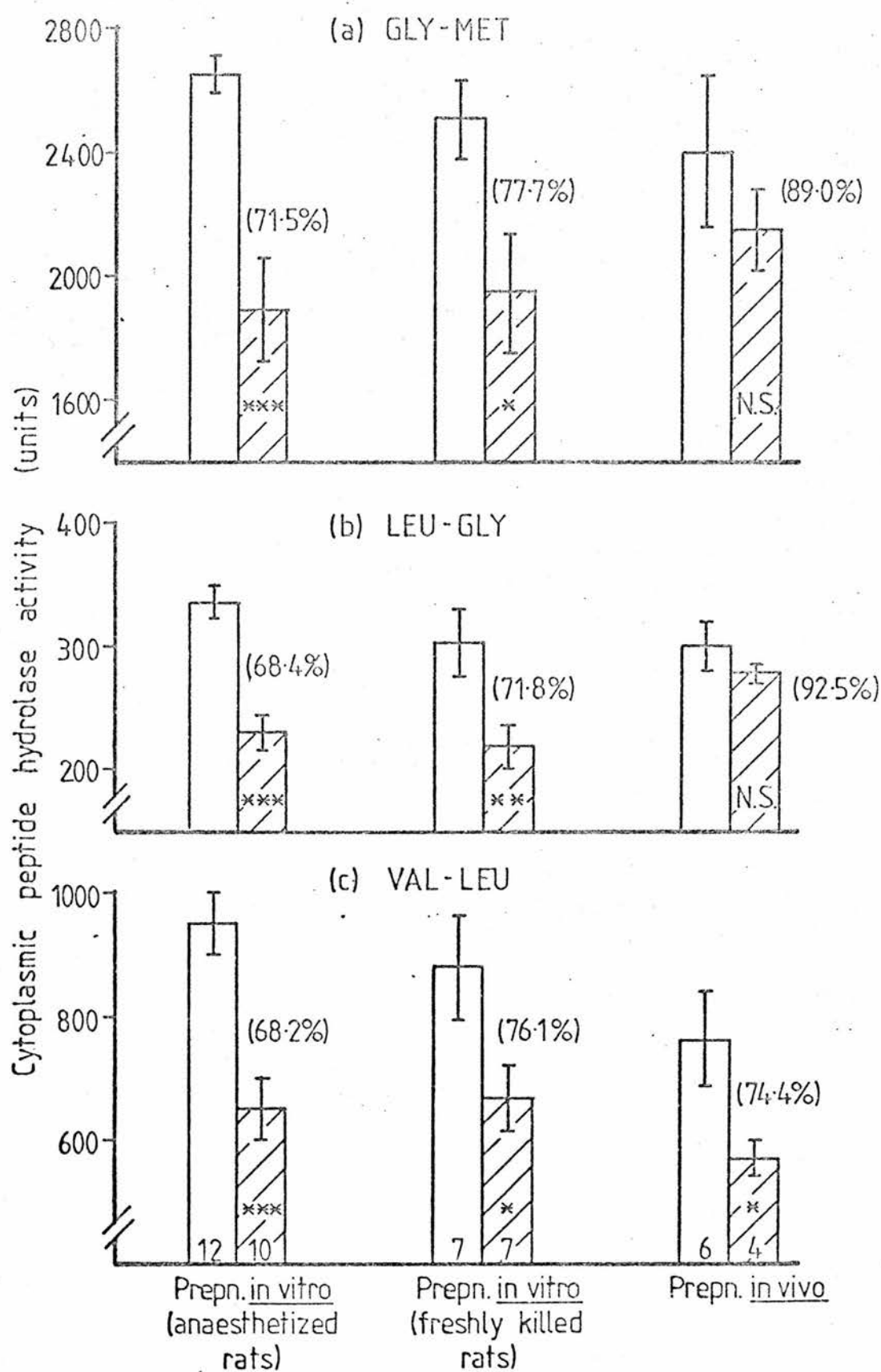
Mucosal DNA content of unperfused and of perfused intestines

Fig. 6.8 shows the DNA content of the mucosa of unperfused intestines and of intestines after perfusion for 1 hour.

In all cases the mucosal DNA content was slightly less (by 6-14%) after perfusion than in the unperfused intestines, although this difference was never significant ($P > 0.05$). However, the difference between the DNA content of the perfused intestines prepared from dead animals and the unperfused ones prepared from ether-anaesthetized rats was significant ($P < 0.05$).

Fig. 6.9 shows the cytoplasmic peptide hydrolase activities expressed as units/mg DNA in unperfused intestines and in intestines after perfusion for 1 hour. Although in all cases there are losses

Fig. 6.7 Cytoplasmic peptide hydrolase activities in mucosa of unperfused small intestine (open bars) and of small intestines perfused for 1 hour in vitro or in vivo (hatched bars). Intestines for perfusion in vitro were prepared both from ether-anaesthetized rats and from freshly killed rats. Substrates were (a) Gly-L-Met, (b) L-Leu-Gly, and L-Val-L-Leu. Values are means \pm S.E.M. and the number of animals is shown within the bars in (c). Values in parentheses show the activities in perfused intestines expressed as percentages of the activities in unperfused intestines. The significance levels of the differences from values in unperfused intestines are indicated thus: ***P < 0.001, **P < 0.02, *P < 0.05, N.S. P > 0.3.



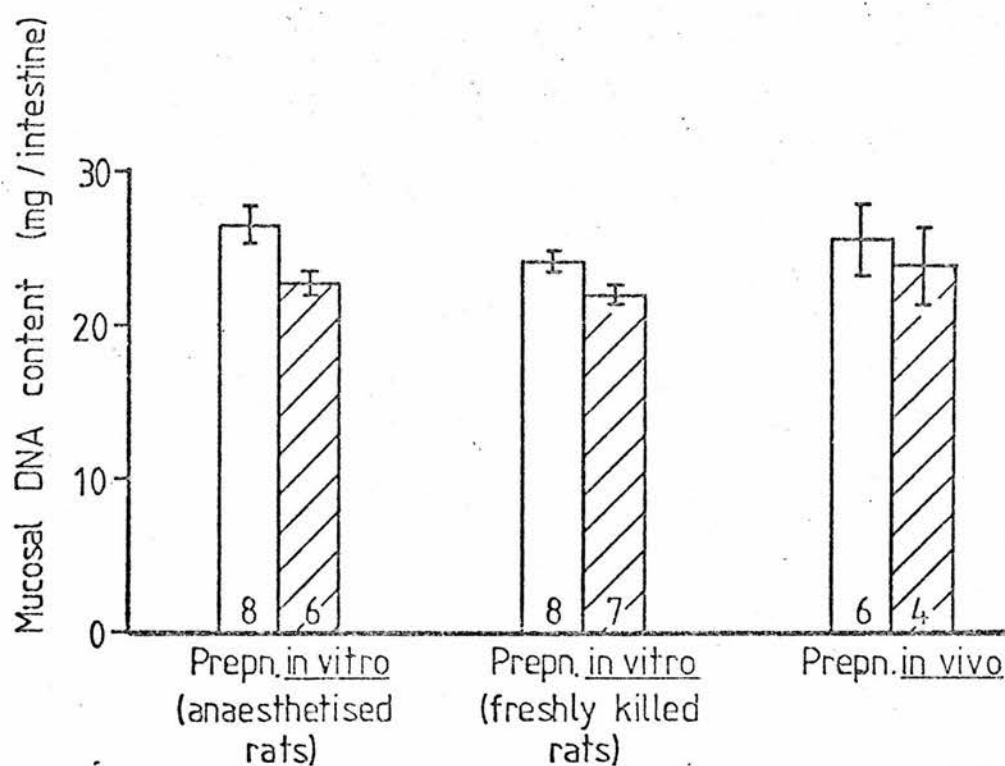
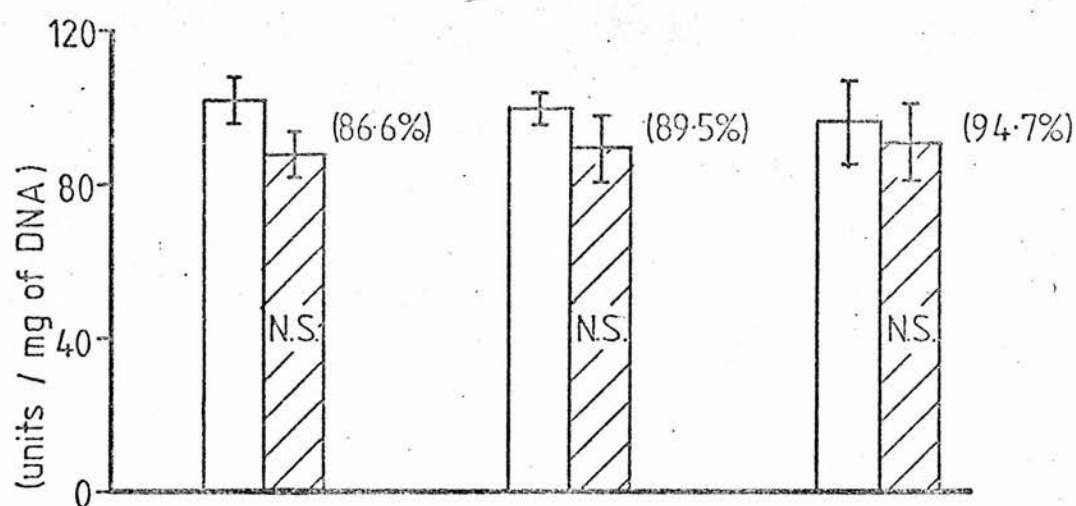


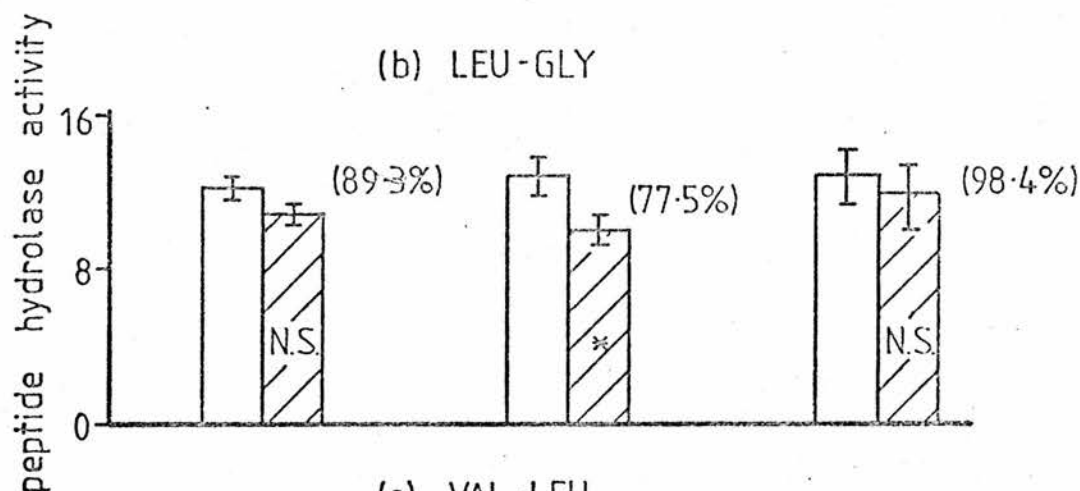
Fig. 6.8 DNA content of whole small intestinal mucosa of unperfused intestines (open bars) and of intestines after perfusion for 1 hour in vitro or in vivo (hatched bars). Intestines for perfusion in vitro were prepared both from ether-anaesthetized rats and from freshly killed rats. Values are the mean \pm S.E.M. and the number of intestines is shown within the bars.

Fig. 6.9 Cytoplasmic peptide hydrolase activities per mg DNA in unperfused small intestine (open bars) and after perfusion for 1 hour in vitro or in vivo (hatched bars). Intestines for perfusion in vitro were prepared both from ether-anaesthetized rats and from freshly killed rats. Substrates were (a) Gly-L-Met, (b) L-Leu-Gly, and (c) L-Val-L-Leu. Values are means \pm S.E.M. and the number of intestines is shown within the bars in (c). Values in parentheses show the activities after perfusion for 1 hour expressed as a percentage of activities in unperfused intestines.

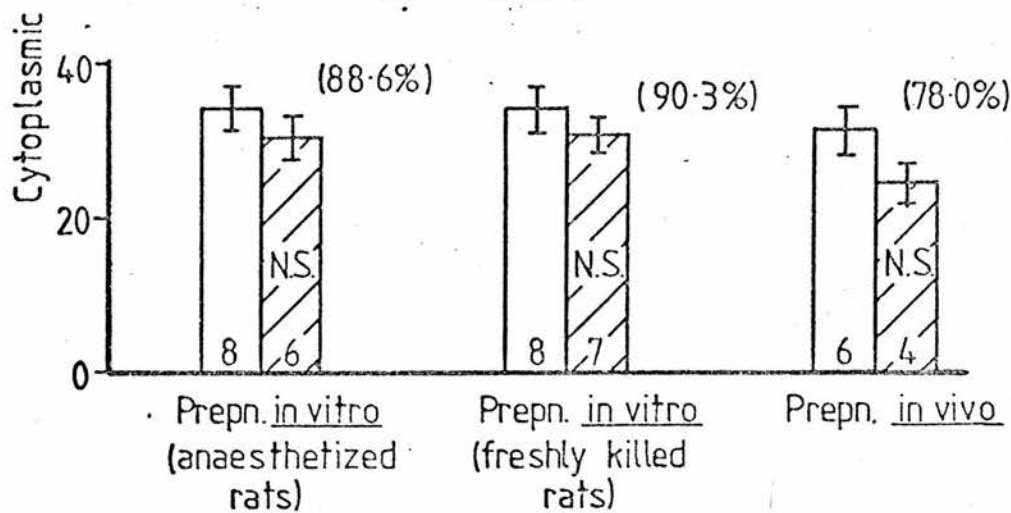
(a) GLY-MET



(b) LEU-GLY



(c) VAL-LEU



of enzymic activity, relative to DNA content, during perfusion they are not significant ($P > 0.1$) except for L-Leu-Gly peptidases from the killed animals ($P < 0.05$).

Total recovery of peptide hydrolase activities after perfusion

The total peptidase activities recovered in luminal effluents, serosal secretions and mucosal cytoplasm after perfusion for 1 hour are shown in Table 6.1 as a percentage of the activities in mucosal cytoplasm of unperfused intestines.

Table 6.1 Total peptidase activities recovered in luminal effluents, serosal secretions and mucosal cytoplasm after perfusion in vitro and in vivo for 1 hour

Preparation	Number of intestines	% of activity in unperfused intestines		
		Gly-Met	Leu-Gly	Val-Leu
<u>In vitro</u> from anaesthetized rats	10	72.4	70.9	69.4
<u>In vitro</u> from freshly killed rats	7	84.1	81.4	84.0
<u>In vivo</u>	4	90.3	95.8	76.2

The amount of activity recovered after perfusion was least when intestines removed from anaesthetized rats were perfused in vitro. However, as shown in Fig. 6.7, this is due partly to the fact that the peptide hydrolase activities found in unperfused intestines removed from ether-anaesthetized rats were greater by 6-25% than those found in unperfused intestines removed from freshly killed rats or from urethane-anaesthetized rats.

DISCUSSION

These results extend earlier studies (Silk and Kim, 1975; Lindberg, 1972) which demonstrated that intestines in vitro can release peptide hydrolase enzymes into perfusion and incubation media, but, since different activities have been measured, direct comparisons between studies cannot be made. However, the results indicate that the amounts appearing in the luminal effluent of the preparation used in this work are normally very small, amounting over 1 hour to only 0.6 - 1.9% of the initial cytoplasmic content (Fig. 6.6). The enzyme activities recovered in the luminal effluent during perfusion in vitro and in vivo were closely similar provided that the preparation in vitro was set up from anaesthetized animals - a practice which is rigorously adopted in this laboratory. However, when the segments for perfusion were excised from freshly killed animals, the enzyme activities found in the luminal effluents were four to eight times greater than when the animals were maintained under ether anaesthesia while the intestines were being connected to the perfusion apparatus (Fig. 6.6). Similarly, the activities found in the secretion on to the serosal surface of the intestine were small (0.3 - 0.5% of the initial cytoplasmic activity) when intestines were set up from anaesthetized rats. These values also increased substantially (three-fold) when animals were killed before removal of the intestine (Fig. 6.6). The results from the preliminary study (Fig. 6.2) indicate that the release of enzymes into the luminal effluent was a continual process at an approximately constant rate throughout the hour of perfusion rather than one which commenced after a certain time.

Thus, these results indicate that the release of hydrolases from the perfused intestine is normally small although it can be increased experimentally. Although this might suggest that the release of peptidases is not a normal physiological process, it must be noted that the amount of peptidase activity within the mucosa is so large that the release of 1% cannot necessarily be dismissed as functionally insignificant, especially in view of the likelihood that hormones stimulate this process. Cholecystokinin-pancreozymin has been shown to stimulate a five to nine fold increase in the release of brush border enzymes from the rat small intestine perfused in vivo (Götze et al, 1972). Eloy et al (1978) reported that pentagastrin, caerulein and glucagon stimulate a four to eight fold increase in the release of aminopeptidase activity from rat small intestine perfused ex vivo. A five to nine fold stimulation of the release observed with the preparation in vivo might result in significant luminal activity.

The high rates of peptidase release in vitro, which Lindberg (1972) and Silk and Kim (1976) reported, probably had been influenced by the method of preparation. It must be emphasised that the intestines removed from freshly killed rats in the experiments described in this chapter were deprived of an oxygen supply for only about 4 minutes. This is the time between the death of the rat and the establishment of luminal perfusion with the segmented flow of oxygenated perfusate and includes the period during which the lumen is washed out with oxygenated saline. Silk and Kim (1976) studied the release of peptidase activities from intestinal segments incubated in vitro in saline with no additional oxygen supply apart from that dissolved in the saline throughout the 90 minute incubation period.

Although it is not known that results similar to those obtained in this study would be obtained from all other types of intestinal preparation in vitro, or whether temporary anoxia specifically causes the enzyme release, the results indicate the merit of using anaesthesia and the precautions of Fisher and Parsons (1949) and Fisher and Gardner (1974) for the preparation of isolated intestines for perfusion, especially during studies on peptide absorption* and hydrolysis or on peptide hormones. Furthermore, the similarity observed between the peptidase release from intestines perfused in vitro by the method of Fisher and Gardner (1974) and from intestines perfused in vivo lends further support to the value of these precautions. Also, the absorptive activity of isolated perfused intestine prepared from anaesthetized rats is substantially greater than if the animals are killed before removal of the intestine (Gardner, 1978).

The relatively large amount of activity against Gly-L-Met found in the luminal effluent and serosal secretion (Fig. 6.6) reflects the large amount of Gly-L-Met activity in the cytoplasm (Fig. 6.7); this is probably not due to differences in the stability of the three activities studied since Gly-L-Met hydrolase activity is the most labile (see Chapter 4, Fig. 4.8).

Silk and Kim (1976) concluded that the peptidase release in vitro by their preparation was not affected by the incubation

* Wiggans and Johnston (1959) observed that the serosal fluid of everted sacs of rat intestine contained peptidase activity and stressed that this made it impossible to interpret whether intact peptides had crossed the intestine.

temperature. However, both of the enzymic activities studied by Silk and Kim (1976) are thermolabile; cytoplasmic activities against Gly-L-Phe and L-Phe-Gly are decreased to less than 20% following incubation at 25°C and 37°C respectively (Heizer et al, 1972). It is therefore probable that during incubation of intestinal tissue at 37°C the estimated release of peptidase activity will be much lower than the true value due to heat inactivation of the enzymes during the course of the experimental period, a point which escaped Silk and Kim's attention. Thus, in view of the thermolability of the activities estimated by these authors, they were not justified to conclude that peptidase release in vitro is not affected by the incubation temperature.

The total recovery of peptidase activities after perfusion is fairly low and depends upon the preparation used (Table 6.1); lowest recoveries were observed when intestines, removed from anaesthetized rats, were perfused in vitro and the highest recoveries were obtained with intestines perfused in vivo. However, these results do not indicate an increased loss of peptidase activities from intestines removed from anaesthetized rats and perfused in vitro since the activities in the unperfused intestines were greater than those from the other two preparations studied (Fig. 6.7). Furthermore, there was a substantial increase in the activities recovered when care was taken to minimize heat inactivation of the peptide hydrolase activities. The discrepancy between the activities in unperfused intestines and those recovered after perfusion cannot be explained, but it might be due to heat inactivation of the peptide hydrolase activities during perfusion. If so then the activities released into the luminal effluents and serosal secretions might have been

underestimated.

It is not known whether the enzymes released into the lumen are of cytoplasmic or brush border origin: however, the work of Silk and Kim (1976) and Silk et al (1976) suggests that they may be predominantly cytoplasmic with some contribution by ileal brush border.

DNA is also lost (6 - 14% in 1 hour) from the perfused intestines (Fig. 6.8). The small DNA loss from intestines perfused in vitro accords with the good absorptive stability of the preparation reported by Fisher and Gardner (1974) (see also Chapter 3). However, in the experiments in vitro at least, the losses of enzyme activity from the cytoplasm during perfusion were proportionately slightly greater than the losses of DNA (Fig. 6.9). Although this difference is not quite significant it was consistently observed in all three groups of experiments; hence it is probable that enzymes are lost from the mucosa by a combination of sloughing of whole (but not necessarily intact) cells (leading also to the slight DNA loss) and a more specific process. This is consistent with Lindberg's (1972) observation that disaccharidase activities were not released in vitro along with the dipeptidase activities and with the substantially larger amounts of Gly-L-Leu hydrolase activities, expressed per mg nitrogen, found in saline in the intestinal lumen after incubation in vitro than in vivo (Lindberg et al, 1975, p. 228).

Although the results of this study demonstrate the stability of intestines perfused in vitro by the technique of Fisher and Gardner (1974) it must be noted that there is a small but consistent loss of both peptide hydrolase activities and mucosal DNA during perfusion.

Thus, it must be concluded that intestines, after perfusion, cannot be used for accurate estimations of mucosal peptide hydrolase activities and DNA contents and consequently, that these two measurements and that of absorption cannot be made on the same intestine. This is a great disadvantage since the use of two sets of animals introduces greater variability into the results and also prevents direct comparisons between the three measurements. Furthermore, these results indicate that intestines used for the estimation of peptide hydrolase activities and mucosal DNA contents should, ideally, be removed from anaesthetized rats since there is a slight, though not significant, loss of both peptidase activities and DNA when animals are killed immediately before removal of the intestine. However, there is also a slight loss of peptidase activities and of DNA when animals are anaesthetized with urethane as opposed to ether prior to removal of the intestine. This difference cannot be explained but it is of note that the induction of anaesthesia with urethane is complete after about an hour whereas ether anaesthesia is induced within minutes.

These results stress that the characteristics of the preparation are strongly dependent upon the method of preparation of the intestine for perfusion. They raise the possibility that preparations in which the precautions advocated by Fisher and Parsons (1949) and by Fisher and Gardner (1974) cannot be observed (e.g. everted sacs and intestinal rings) may give misleading results, or at any rate, different results, due to the presence of peptide hydrolase activities in the incubation medium. This may be especially relevant in studies on absorption of peptides and on the effects of peptide hormones.

Peptide hydrolase activities against Gly-L-Met and L-Val-L-Leu estimated in the mucosal cytoplasm by the method of Nicholson and Kim (1975) were much greater than those estimated in the preliminary study by the method of Fujita et al (1972) as modified by Gardner et al (1978) (Figs. 6.3 and 6.7). This demonstrates clearly the benefits of the final choice of peptide hydrolase assay and of the experimental design adopted in Part II of this study. Furthermore, it was shown in Chapter 4 that peptide hydrolase activity against Gly-L-Met is the most thermolabile of the three enzyme activities estimated and it is the estimates of this activity which show the greatest increase upon introduction of the precautions to minimize heat inactivation of peptide hydrolase activities (compare Figs. 6.1 and 6.6, and Figs. 6.3 and 6.7). However, the converse is true for the hydrolase activity against L-Leu-Gly; these were less when estimated in the mucosal cytoplasm by the method of Nicholson and Kim (1975) than those estimated by the method of Fujita et al (1972) as modified by Gardner et al (1978) (Figs. 6.3 and 6.7). Also, cytoplasmic peptide hydrolase activities against Gly-L-Met and L-Val-L-Leu, estimated by the method of Fujita et al (1972), were greater when intestines were removed from urethane-anaesthetized rats than when intestines were removed from ether-anaesthetized animals (Fig. 6.3) but were less when estimated by the method of Nicholson and Kim (1975) (Fig. 6.7). These are weak points in this study and the differences cannot be explained.

CHAPTER 7

Does intestinal ischaemia affect plasma peptide hydrolase activities?

Introduction

Although acute mesenteric vascular occlusion with intestinal ischaemia is a relatively uncommon clinical occurrence, the resulting mortality is very high (Harper and Buist, 1978; Watt *et al*, 1967). This is largely due to the difficulty in diagnosis of the condition because of the lack of specific symptoms in its early stages. Early diagnosis is essential since successful treatment can only be accomplished by surgery before the recognisable specific symptoms become apparent (Koehler and Kressel, 1978; Morris *et al*, 1962).

The intestine is supplied with blood from the splanchnic circulation via three great vessels, the coeliac axis and the superior and inferior mesenteric arteries. An extensive network of collateral vessels ensures an adequate blood supply to the intestine, most regions being supplied by more than one of the great vessels. Intestinal ischaemia is therefore a rare event (Boley *et al*, 1978, p. 7). However, since the superior mesenteric artery accounts for a major part of the splanchnic blood flow, occlusion of this vessel is one of the two most common causes of intestinal ischaemia (Jacobson, 1970; Marston, 1977). A reduced mesenteric blood flow due to cardiac failure is the other common cause (Marston, 1977).

Mortality from acute occlusion of the superior mesenteric artery remains at over 90% (Mavor, 1972), not only because of the difficulty

in diagnosis (Harper and Buist, 1978; Mavor, 1972) but also, since the condition is uncommon, because of failure to suspect intestinal ischaemia (Watt et al, 1967). In 1967 there were 653 deaths from mesenteric infarction in the U.K. (Marston, 1970). Jacobson and Lanciault (1979) suggest that in the U.S.A. the death rate from abnormalities of the gastrointestinal circulation may be greater than that from cancer of the same organs.

During the early stages of intestinal ischaemia the symptoms are non-specific, consisting of abdominal pain usually accompanied by vomiting and diarrhoea (Harper and Buist, 1978). The interval between the onset of ischaemia and the development of peritonitis can be of the order of days or weeks, but it is more commonly about six hours and it is during this period that diagnosis must be made (Mavor, 1972).

Both clinical studies and animal experiments have so far failed to find any structural or functional abnormality in the intestine that can be used as the basis of a clinical test to aid the diagnosis of a reduced mesenteric blood flow (Marston, 1977). Elevated serum lactate dehydrogenase has been reported in only 50% of patients with infarction of the small intestine (Zimmerman and West, 1964). In another study all patients with intestinal ischaemia demonstrated elevated serum lactate dehydrogenase, but a consistent elevation was not observed in dogs after ligation of the superior mesenteric artery (Braun et al, 1959). Similarly in dogs and cats serum alkaline phosphatase and glutamic oxalacetic transaminase have been reported to increase (Bonakdarpour et al, 1976; Dagher et al, 1967; Larson et al, 1959; Rudolph et al, 1957) or remain unchanged (Marston et

al, 1969; Rosato et al, 1971) during intestinal ischaemia. These enzyme activities are not specific to intestinal cells and would therefore have limited value in the diagnosis of intestinal ischaemia. Similarly, the raised white cell counts, elevated haematocrit and metabolic acidosis observed in acute mesenteric ischaemia are extremely non-specific (Boley et al, 1978, p. 19).

Angiography has been used with great success in confirming diagnosis of mesenteric ischaemia (Watt et al, 1967). However, this is an expensive and time consuming diagnostic aid and is therefore generally used only after a provisional diagnosis has been made (Boley, 1978, p. 27).

Thus a specific biochemical test that would provide an early and reasonably specific index of intestinal ischaemia would have great potential value. A possible diagnostic test was suggested by the results of the peptidase release study described in Chapter 6. When intestines were removed from freshly killed rats, the dipeptide hydrolase activities appearing in the serosal secretion during perfusion were three to four times greater than when intestines were removed from anaesthetised rats. The intestines removed from the freshly killed rats were deprived of oxygen for less than 4 minutes. The enzyme release was a very rapid response and it is possible that the release was stimulated by this short period of anoxia. In the intact animal enzymes released from the serosal surface of the intestine should enter the mesenteric blood vessels and lymph. Peptide hydrolase activities in the blood are normally very low (Krzysik and Adibi, 1977). If intestinal ischaemia stimulates the release of peptide hydrolase activities into the blood, then the

estimation of peptide hydrolase activities in the blood could possibly be used as a simple and rapid method of diagnosis.

Intestinal ischaemia can be induced experimentally in animals by ligation of the mesenteric blood supply. The severity of ischaemia induced can be pre-determined by the position and number of ligatures. The pathological changes that result from ischaemia induced experimentally in this way are similar to those seen in clinical ischaemia (Boley et al, 1978, p. 11).

Therefore intestinal ischaemia has been induced in rats and subsequently peptide hydrolase activities were estimated in the plasma at various times after ligation; they were compared with values from unligated controls.

METHODS

Ligation of arcadian vessels and the superior mesenteric artery

Female rats weighing 180 - 200 g were anaesthetised throughout the experiment with urethane (1.5 ml of 0.8g/ml solution) injected subcutaneously whilst under temporary light ether anaesthesia. Anaesthesia was complete after 60 - 90 minutes. The abdomen was then opened by a mid-line incision and the small intestine and caecum gently exteriorised. 'Mild' ischaemia was induced by ligation, with silk thread, of a group of arcadian vessels supplying about 10 cm of small intestine commencing approximately 15 cm distal to the ligament of Treitz. The abdominal contents were then replaced and the skin incision closed by three stitches. As a control for this preparation, the same procedure was followed in one animal but the vessels were not ligated.

'Severe' intestinal ischaemia was induced by ligation of the superior mesenteric artery. This vessel was located by blunt dissection at the root of the mesentery adjacent to the colon. The artery was isolated from the superior mesenteric vein and ligated with silk thread. The abdominal contents were replaced and the skin incision closed as before. In one animal the artery was isolated by blunt dissection but not ligated. This animal served as the control for this preparation.

All the animals were kept warm by an overhead lamp during the period of ischaemia.

Estimation of plasma peptide hydrolase activities

Approximately 4 ml of blood was taken by cardiac puncture after

20, 60, 120 or 240 minutes from the time of ligation, and after 240 minutes from the controls. Samples were taken up into 10 ml syringes fitted with a 21 g needle and with the dead space of the syringe and needle filled with heparinised saline (0.1 g sodium heparin, Sigma/10 ml 0.9% w/v NaCl). Some rats died during the experiment and the blood sample was taken immediately after death. Blood samples were centrifuged immediately at approximately 4000 g at room temperature in an M.S.E. bench centrifuge. Peptide hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu were estimated immediately in plasma at a dilution of 1:5 by the modified method of Nicholson and Kim (1975), described in Chapter 4. The experiments were timed such that all the blood samples were taken within a 30 minute period to reduce the possibility of loss of activity in the plasma due to storage.

As a further control, plasma peptide hydrolase activities were estimated in rats that had not been subjected to any surgical treatment; blood samples were taken by cardiac puncture from two ether anaesthetised rats.

Table 7.1 Plasma dipeptide hydrolase activities measured at various
times after the induction of intestinal ischaemia

Position of ligature	Duration of ischaemia (mins)	Peptide hydrolase activity (units/ml)		
		Gly-Met	Leu-Gly	Val-Leu
Control	0	0.16	0.18	0.10
Control	0	0.17	0.20	0.12
Control (a.v.)	0	0.17	0.16	0.10
Control (s.m.a.)	0	0.19	0.21	0.12
a.v.	10 (died)	0.14	0.09	0.12
a.v.	20	0.16	0.20	0.11
a.v.	60	0.14	0.15	0.06
a.v.	82 (died)	0.19	0.19	0.11
s.m.a.	20	0.14	0.19	0.09
s.m.a.	60	0.18	0.19	0.09
s.m.a.	100 (died)	0.18	0.17	0.13
s.m.a.	240	0.18	0.17	0.11

Each line refers to an individual rat

a.v. = arcadian vessel

s.m.a. = superior mesenteric artery

RESULTS

Effect of ischaemia on the intestine

The gross appearance of the intestines of the two preparation controls was normal. In all experimental animals the gross appearance of the intestine at the time of cardiac puncture was similar. In one or two places a region of the intestine of about 3 - 4 cm length was heavily bruised and there were extensive haemorrhagic lesions in the adjacent mesenteric vessels. In most cases there was blood in the intestinal lumen. The general appearance of the intestines agreed closely with that described by Whitehead (1976).

Effect of intestinal ischaemia on plasma peptidase activities

Dipeptide hydrolase activities against Gly-L-Met, L-Leu-Gly and L-Val-L-Leu were estimated in plasma at various times after ligation; the results are shown in Table 7.1.

Small, but significant, peptide hydrolase activities were found in the plasma from control rats. However, it is clear that there was no effect of intestinal ischaemia on plasma peptide hydrolase activities even after 4 hours duration of ischaemia.

DISCUSSION

The results of the study described in Chapter 6 suggested that the release of peptide hydrolase activities from the small intestine is normally very small and that it may possibly be stimulated by anoxia. If the release is stimulated by anoxia, then it seems possible that occlusion of the mesenteric blood supply in the intact animal will result in an increased release of intestinal peptidase activity into the mesenteric blood.

This possibility has now been investigated as the basis of a method for the diagnosis of mesenteric infarction. However, as the results presented in Table 7.1 demonstrate, there is no effect either of duration or severity of intestinal ischaemia on plasma peptide hydrolase activity. It is also of note that the operative techniques did not affect plasma peptidase activities since those of the preparation controls do not differ from the activities observed in the plasma taken from ether anaesthetised rats.

The gross appearance of the intestines at the time of cardiac puncture confirmed that intestinal ischaemia had been induced, since damage appeared as early as 10 minutes after ligation. Since the animals were maintained under anaesthesia throughout the period of ischaemia, the deaths cannot be attributed solely to intestinal infarction - since anaesthesia may make the animals more sensitive to surgery and the results of ligation.

Although this study failed to demonstrate an increase in plasma dipeptide hydrolase activities associated with intestinal ischaemia, it is of note that total occlusion of the superior mesenteric artery

or of a group of arcadian vessels will severely impair the mesenteric blood supply and also the venous and lymph drainage of the intestine. Therefore it is possible that the release of dipeptide hydrolase activities from the serosal surface of the intestine was stimulated in this study but due to absolute obstruction of the mesenteric circulation the enzymes did not enter the general circulation.

Severe intestinal ischaemia was apparent in all animals and most had blood in the intestinal lumen. In the human, the appearance of blood in the intestinal lumen is associated with the later stages of the acute disease (Mavor, 1972) and diagnosis must be made before this stage of the disease is reached. Thus it is possible that the method of induction of ischaemia in this study was too severe. Perhaps a less severe induction of ischaemia in the rat would be associated with raised plasma peptide hydrolase activities.

Thus the negative outcome of this experiment should not be regarded as the final verdict as to the potential use of peptidase activities in clinical diagnosis: further studies are justified, especially in view of the benefits at stake.

Very low peptide hydrolase activities (about 0.15 $\mu\text{mol}/\text{min}/\text{ml}$) were found in plasma from control rats. Similarly, Krzysik and Adibi (1977) found no activity against Gly-Gly and activities against Gly-L-Leu amounted to less than 100 $\mu\text{mol}/\text{min}$ in the total blood volume of the rat. Although these activities are very low, their presence might explain the absence of dipeptides from blood.

CHAPTER 8

The differential effects of 5-fluorouracil and starvation on the rat small intestine.

Introduction

In view of the clinical evidence that 5-fluorouracil administration can be associated with major gastrointestinal disturbances, it is desirable to investigate the pathogenesis of these in animals before attempts are made to minimize these disturbances. Although there is clear evidence of severe impairment of intestinal digestive and absorptive functions following administration of 5-fluorouracil, previous studies have not included all the necessary control experiments. Detailed information on several aspects, as discussed below, is therefore essential.

Decreased intestinal absorption following 5-fluorouracil injection has already been demonstrated in the rat (Gardner et al, 1978; Levin, 1968; Roche et al, 1970); histological changes and impairment of enzyme activities in the intestine have been reported both in rat and man (Bounous et al, 1971a and b; Hartwich et al, 1974; Roche et al, 1970). Although it is possible that these effects can be directly ascribed to the 5-fluorouracil, it must be noted that administration of 5-fluorouracil to rats is followed by a severe reduction in food intake (Bounous et al, 1971a; Gardner et al, 1978) and that food restriction or starvation per se are both known to cause changes in the absorptive and enzymic activities of the small intestine (Debnam and Levin, 1975; Kershaw et al, 1960; Kim et al, 1973; Lis et al, 1972; McManus and Isselbacher, 1970; Newey et al, 1970). Hence it is important to dissociate the primary effects of the drug from the secondary ones, i.e. those merely due

to the reduced food intake. Furthermore, the nature of the changes due to the effects of starvation and food restriction is unclear, since the literature contains much apparently conflicting information. There is an extensive literature on this subject but the various studies have used a number of different methods and regions of the intestine for the estimation of both intestinal absorption and enzyme activities. Also, and in particular, absorption has been expressed with reference to many different reference systems such as unit dry or wet weight of small intestine or mucosa, unit length of intestine, whole everted sac, mucosal DNA content, etc. Therefore comparisons between the various results are difficult and the conflicting results may be due, at least in part, to this use of different reference systems.

Therefore the differential effects of 5-fluorouracil and starvation on absorption from the small intestine have been studied with a view to dissociating the effects of 5-fluorouracil on the small intestine from those of the consequent reduced food intake. Intestinal water absorption rates were measured at various times following 5-fluorouracil injection; absorption rates were also measured in uninjected rats subjected to a food restriction regime designed to mimic the food intakes of 5-fluorouracil-injected rats. It must be stressed that such a food restriction regime cannot mimic exactly the feeding pattern of rats following 5-fluorouracil administration. Although the total daily food intake was determined, the feeding pattern during each 24 hour period was not known. Thus the daily food intake was the same as that of 5-fluorouracil-injected rats, but the time at which the food was consumed was probably not the same.

In order to clarify the effects of starvation and food restriction on intestinal absorption and to determine the effect of the use of different reference systems, four different reference systems have been used: whole small intestine, unit length of intestine, intestinal dry weight and mucosal DNA content.

The changes in absorption rate following administration of 5-fluorouracil may result from either (a) a change in the number of absorbing cells, (b) a change in the absorptive capacity of individual absorbing cells, or (c) a combination of these. In this respect Batt and Peters (1976) suggested that absorption rates should be expressed per enterocyte as an indication of the absorptive capacity of each enterocyte. The total number of enterocytes would be determined, they proposed, by estimation of the DNA content of a mucosal homogenate. They suggested that this would enable comparisons between absorption rates estimated from different regions of the intestine and between normal and experimentally altered intestines. However, it must be recognised that the intestinal epithelium consists of an heterogeneous population of cells in which only the mature enterocytes at the tips of the villi are responsible for absorption. Therefore estimation of the mucosal DNA content, though an index of the total cell numbers, is not, as implied by Batt and Peters (1976), a specific index for absorbing cells. This will be exemplified below by the results of the effects of 5-fluorouracil on the intestine.

During the course of the work for this thesis the suppliers of the 5-fluorouracil (Roche Products Ltd) changed the salt form of the drug supplied. Initially the Tris salt of 5-fluorouracil was supplied from out-dated stock. In November 1978, Roche ceased

marketing the Tris salt of 5-fluorouracil in the U.K. and introduced the sodium salt. Administration of the Tris salt of 5-fluorouracil to rats was, without exception, followed within a few hours by obvious signs of severe toxicity which occasionally resulted in death. In contrast, administration of the sodium salt of 5-fluorouracil at the same dose had no effect on the gross appearance of the rats and was never associated with mortality. It is not clear why Roche changed the salt form of 5-fluorouracil. They stated that there were problems with the stability of 5-fluorouracil at a concentration of 50 mg/ml but gave no details, and it is of note that the sodium salt of 5-fluorouracil is available in the U.S.A. at a concentration of 50 mg/ml (Calabresi and Parks, 1975). However, in the U.K. the Tris salt of 5-fluorouracil was supplied at a concentration of 50 mg/ml. Since Roche began marketing the sodium salt of 5-fluorouracil in the U.K. they do not appear to have received any other reports of a difference in the toxicities of the two salts of 5-fluorouracil. It was, therefore, of great interest to study further the differential toxicities of the two salts.

Although there are obvious differences in the systemic toxicities of the Tris and sodium salts of 5-fluorouracil and in the effects on body weights and food intakes, the changes in water absorption rates following administration of either salt were found to be very similar. The differential effects of the Tris and sodium salts of 5-fluorouracil on intestinal absorption are therefore included in this chapter since they offer a further means of dissociating the effects of 5-fluorouracil on the intestine from those of the reduced food intake.

There are several possible reasons for the severe toxicity of

the Tris salt of 5-fluorouracil and some of these have been studied. A difference in the pharmacokinetics of the two salts of 5-fluorouracil is discussed in the next chapter (Chapter 9). A matched Tris placebo was obtained from Roche and administered to rats in order to determine whether the toxicity was due to the Tris base rather than to the 5-fluorouracil. The Tris salt has a very high osmolality and is supplied at twice the concentration of the sodium salt of 5-fluorouracil. Therefore the effects of the concentration and osmolality of the 5-fluorouracil injection on the gross appearance of rats and on intestinal absorption have been studied. As a further control experiment the effects of temporary light ether anaesthesia, as used during administration of 5-fluorouracil, on intestinal absorption have been determined since ether itself is known to stress animals (Martindale, 1977, p. 696; The Pharmaceutical Codex 1979, p. 337).

The majority of the results presented in this chapter were obtained during the period before the sodium salt of 5-fluorouracil was available. Therefore the effects of food restriction were based on the observation of food intakes of female rats after administration of the Tris salt of 5-fluorouracil. During the course of the work for this thesis it became clear that there were several advantages to be gained from the use of male rats. These were concerned with ease of handling and the adaptability of male rats to dietary modification. Therefore the differential toxicities of the two salts of 5-fluorouracil were studied in male rats rather than in female rats. However, results will be presented which suggest that the intestinal toxicity of 5-fluorouracil is independent of the sex of rat used. Thus it is justifiable to compare absorption results

from male and female rats. It must be noted, however, that the food intakes and growth rates of male rats are much greater than those of female rats and therefore effects on body weights and food intakes should only be compared within a single sex.

A paper containing the results presented in this chapter has been published (Gardner and Plumb, 1981a).

METHODS

5-Fluorouracil administration

Male and female rats weighing 160 - 200 g were injected intraperitoneally whilst under temporary light ether anaesthesia with either the Tris (50 mg/ml, 384.3 mmol/l) or the sodium (25 mg/ml, 192.2 mmol/l) salt of 5-fluorouracil at a mean dose of 1.44 mmol/kg body weight unless otherwise specified. All injections were given between 10:00 and 10:30.

As controls for the injected rats, 8 rats were exposed to temporary light ether anaesthesia only and another 8 rats received an injection of a matched Tris placebo (Roche Products Ltd) whilst under anaesthesia. The volume of placebo administered was equal to the volume containing the appropriate dose of the Tris salt of 5-fluorouracil.

All rats were caged in groups of 4 before and after injection and daily food intakes, body weights (measured between 10:00 and 10:30) and the incidence of diarrhoea were noted.

Experimental procedure

Intestinal water absorption rates and dry weights (for method see Chapter 3) were estimated in control uninjected rats and on either the first or the third day after injection or exposure to ether, except in one experiment in which they were estimated on each of the first 5 days after injection of the Tris or sodium salts of 5-fluorouracil.

The mucosal DNA content was estimated by the method described

in Chapter 5 in control (uninjected) rats and on the first and third days after administration of the Tris or sodium salts of 5-fluorouracil.

Osmolarity estimation

The osmolarities of the two salts of 5-fluorouracil were estimated on 0.5 ml samples in an 'Osmette S Automatic osmometer' (Precision Systems, Waltham, Massachusetts). The sodium salt was used undiluted and the Tris salt was used both undiluted and diluted 1:1 with distilled water. The osmometer was calibrated with two commercial standards (100 and 500 mOsm/l) before use.

Food restriction

In order to determine the effects of the reduced food intake following 5-fluorouracil administration the food intake of a group of rats was restricted to that observed during the first 3 days following injection of 5-fluorouracil. These experiments were carried out before the sodium salt of 5-fluorouracil became available and therefore refer only to the Tris salt of 5-fluorouracil in female rats.

In a preliminary experiment the daily food intakes of 8 rats (2 cages of 4 rats) were recorded on each of the first 3 days after 5-fluorouracil (Tris salt) administration. The food intakes of a further group of rats was restricted to these observed food intakes of 0, 0, and 5.4 g/rat for the first, second and third days respectively.* For this experiment rats were caged in groups of 4

*The regime adopted differs slightly from the food intakes shown in Fig. 8.7 since this figure includes all observations on food intakes whereas the food restriction regime was based only on a preliminary experiment.

for the first 48 hours and then caged individually and given 5.4 g of the Oxoid diet each. Coprophagy was not prevented but cages and bedding were changed daily during food restriction in order to minimise it.

Some rats were fasted for 24 hours and used either for measurement of intestinal water absorption and dry weight or for mucosal DNA estimation. Others were fasted for 48 hours and then given 5.4 g of the Oxoid diet during the next 24 hours. These rats were then used on the third day of food restriction for the estimation of intestinal absorption, dry weight and mucosal DNA contents.

This regime is referred to throughout the chapter as the food restriction regime.

Estimation of mucosal DNA content

Since there is a small loss (about 16% per hour) of mucosal DNA during luminal perfusion, DNA estimations cannot reliably be carried out on intestines after perfusion (see Chapter 6). Therefore the mucosal DNA content was estimated on unperfused intestines; hence estimates of water absorption and mucosal DNA content were not available for the same intestine. Absorption per mg DNA was therefore calculated from the ratio of the mean absorption rate per whole small intestine and the mean mucosal DNA content. The variance of a ratio can be calculated by the approximation given by Cornish-Bowden (1976, p. 173). If the covariance of the numerator and denominator is assumed to be zero this simplifies as follows:

$$\text{variance} \left| \frac{x}{y} \right| = \frac{\text{variance } (x)}{\frac{2}{y}} + \frac{\bar{x}^2}{\frac{4}{y}} \frac{\text{variance } (y)}{\frac{3}{y}} - \frac{2\bar{x} \text{ covariance } (xy)}{\frac{3}{y}}$$

If covariance $(xy) = 0$

Then

$$\text{variance}\left(\frac{x}{y}\right) = \frac{\text{variance}(x)}{\frac{\bar{y}^2}{y}} + \frac{\bar{x}^2 \text{variance}(y)}{\frac{\bar{y}^4}{y}}$$

This formula probably over-estimates the variance since it is assumed that absorption rates and mucosal DNA contents do not vary systematically together and hence that the covariance term is zero. This assumption was necessary since the covariance is unknown and could not be estimated. However, it must be noted that this error will tend to under-estimate the significance of any differences.

RESULTS

Gross appearance of rats following administration of 5-fluorouracil

Within a few hours after injection of the Tris salt of 5-fluorouracil all rats became very cold and lethargic with obvious respiratory distress. In some cases the toxicity was so severe that death resulted, usually within the first 24 hours after treatment. The earliest deaths occurred about 5 hours after injection. These toxic symptoms continued through the second and third days after injection by which time most of the rats had developed diarrhoea. The diarrhoea was transient, seen only on the third and fourth days after administration of the drug. By the fifth day after 5-fluorouracil injection the obvious signs of toxicity were absent and the gross appearance of the rats was normal.

Internally, severe macroscopic haemorrhagic lesions on the surface of the pancreas were noted on the third day after injection.

In contrast, administration of the sodium salt of 5-fluorouracil at the same molar dosage had no effect on the gross appearance of rats and was never associated with mortality. Diarrhoea and the consequent malaise was present on the third and fourth days after injection but otherwise none of the toxic symptoms observed in rats injected with the Tris salt of 5-fluorouracil were noted.

Mortality

Table 8.1 summarises the mortalities after administration of 5-fluorouracil for all the rats used in experiments included in this

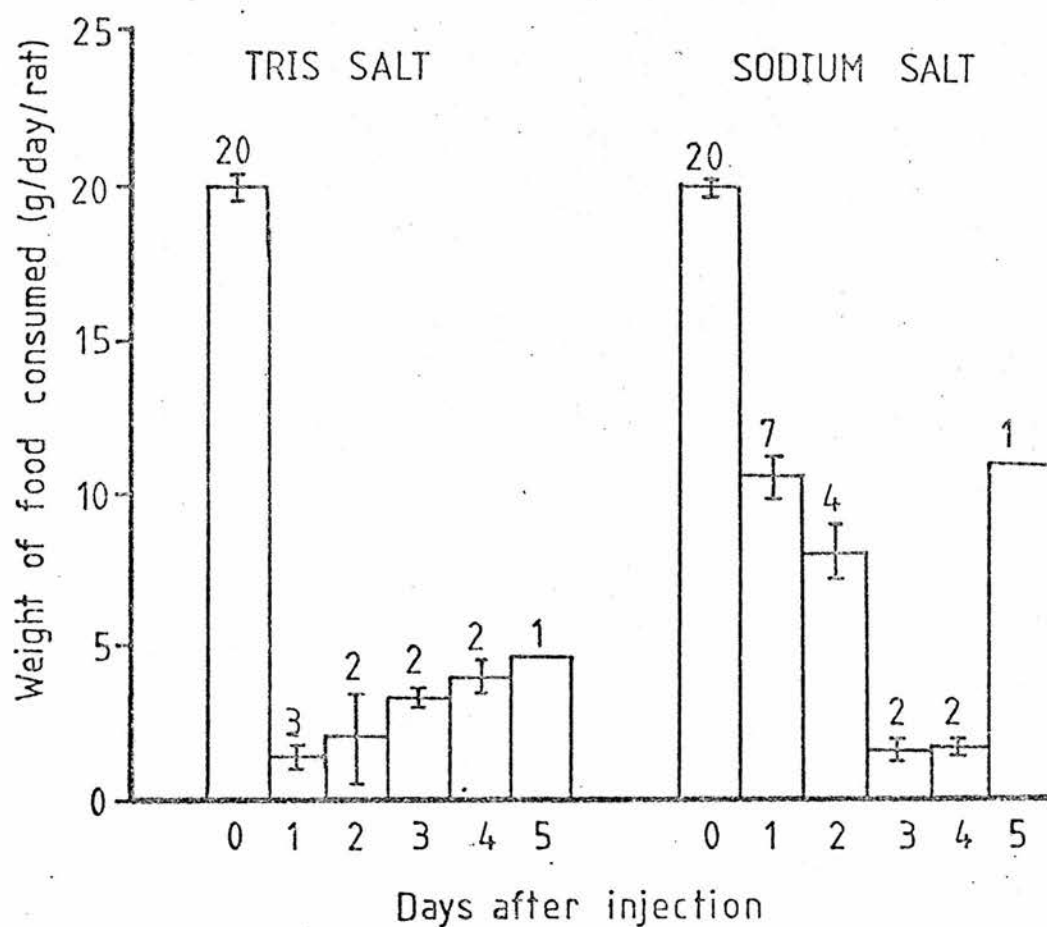


Fig. 8.1 Daily food intakes of control, uninjected, male rats (Day 0) and of male rats during the first 5 days following administration of either the Tris or the sodium salt of 5-fluorouracil. Values are the mean \pm S.E.M. and the number of observations on groups of 4 rats is shown above the bars.

thesis.* Results from rats fed on an 'elemental' diet are included since these are similar to those from rats fed on a standard diet (see Chapter 10).

Table 8.1 Mortality in male and female rats during the first 3 days after administration of the Tris or sodium salt of 5-fluorouracil

Sex	Salt of 5-fluorouracil	Total No. injected	Total deaths	%Mortality
Female	Tris	177	21	11.9
Male	Tris	142	27	19.0
Female	Sodium	13	0	0
Male	Sodium	436	0	0

Whilst a significant mortality was observed in both male (19.0%) and female (11.9%) rats following administration of the Tris salt of 5-fluorouracil, no deaths were observed after injection of a total of 449 rats with the sodium salt of 5-fluorouracil.

Effects of 5-fluorouracil on food intakes

Fig. 8.1 shows the food intakes of control, uninjected, rats and on each of the first 5 days after administration of either the Tris or sodium salt of 5-fluorouracil. Male rats were used. During the first 24 hours after injection of the Tris salt of 5-fluorouracil the food intake decreased to only 7% of the normal daily intake. Then there was a gradual increase in the daily food intakes over the next

*excluding those used for pharmacokinetics experiments since these were also subjected to prolonged anaesthesia and surgery.

few days such that by the fifth day after 5-fluorouracil administration the total food intake over the 5 day period was 16% of the normal intake over the same period.

In contrast, when the sodium salt of 5-fluorouracil was administered the food intake during the first 24 hours after injection decreased only to 50% of the normal daily intake and had only decreased to 7% of normal by the third day after injection. The total food intake during the first 3 days after administration of the sodium salt of 5-fluorouracil (34% of the normal intake) was three times that after administration of the Tris salt of 5-fluorouracil (11% of the normal intake). The food intake increased to 55% of the normal daily intake on the fifth day after administration of the sodium salt: thus the total food intake over the 5 day period (34% of the normal intake) was twice that observed after administration of the Tris salt of 5-fluorouracil.

Effects of 5-fluorouracil on body weights

Fig. 8.2 shows the body weights of male rats on each of the first 5 days after administration of either the Tris or the sodium salt of 5-fluorouracil. Although the body weight of all rats was decreased by about 20 - 30 g by the third day after 5-fluorouracil administration, no statistically significant effect on body weight would be observed if the mean body weight of all the rats was compared by an unpaired test, since the initial body weights ranged from 160 - 200 g. However, when the body weight was expressed as a percentage of the body weight on the day of injection significant effects on body weights were observed.

The body weights decreased on each of the first 4 days after

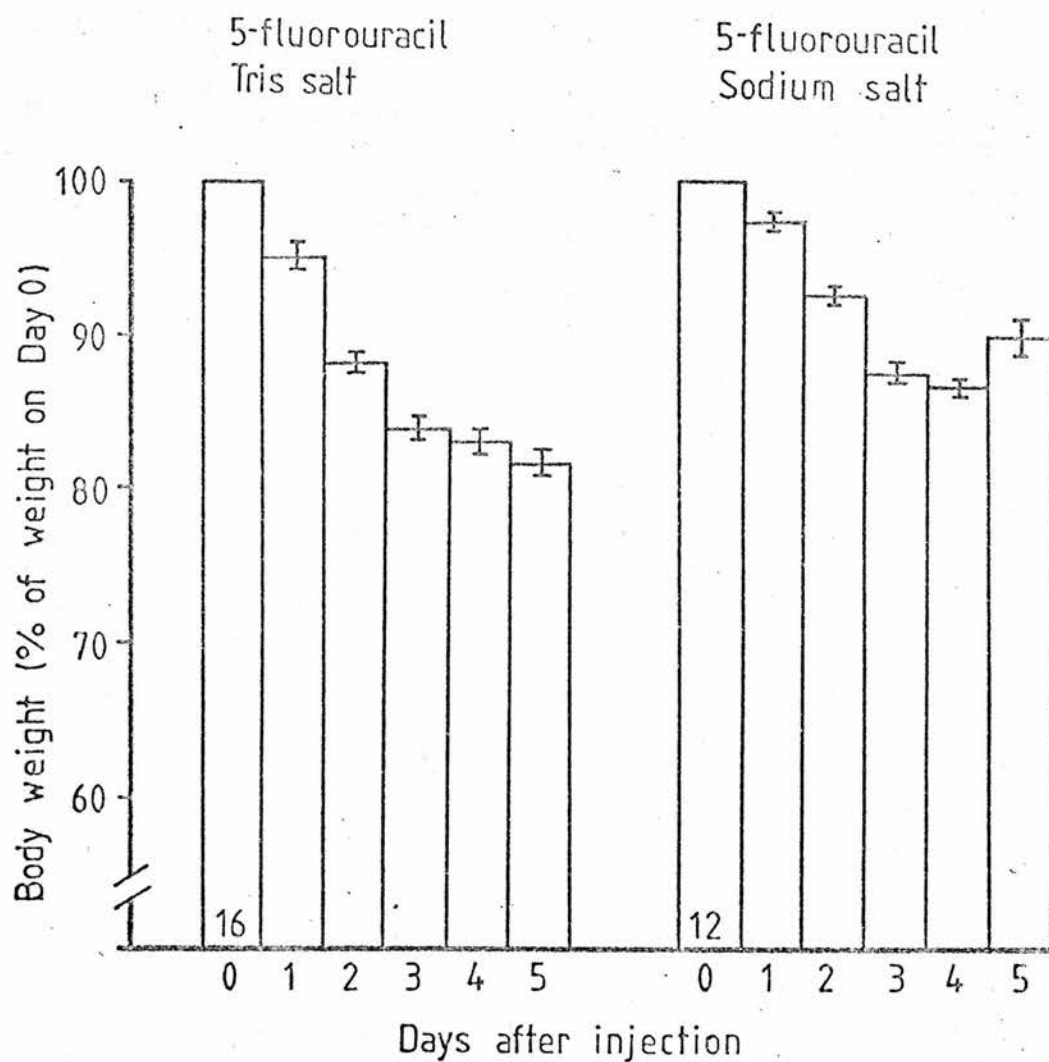


Fig. 8.2 Body weights of male rats on each of the first 5 days after administration of either the Tris or sodium salt of 5-fluorouracil. Values are the mean \pm S.E.M. of the weights expressed as a percentage of the body weight on the day of injection (Day 0). Numbers of observations, each on a single animal, are shown within the bars for Day 0.

injection of either the Tris or the sodium salt of 5-fluorouracil. On the fifth day after administration of the Tris salt the body weights decreased further, whereas they increased on the fifth day after administration of the sodium salt of 5-fluorouracil. On each of the first 4 days after 5-fluorouracil administration the body weight losses were consistently significantly greater after administration of the Tris salt than after administration of the sodium salt ($P < 0.02$).

Intestinal water absorption rates and dry weights on each of the first five days after administration of the Tris or sodium salt of 5-fluorouracil

Figs. 8.3 and 8.4 show the water absorption rates and dry weights respectively of intestines from control male rats and on each of the first 5 days after administration of the Tris and sodium salts of 5-fluorouracil in male rats. Changes in both water absorption rates and dry weights were similar after injection of each salt of 5-fluorouracil. Water absorption rates were unchanged on the first day after 5-fluorouracil injection but had decreased to about 26% of the control rates (i.e. in uninjected rats) by the third day. By the fifth day the absorption rates had increased to about 70% of the control rates.

In contrast, there was a rapid decrease in intestinal dry weight to about 50% of that of control intestines from uninjected rats by the first day after 5-fluorouracil injection. There was a further decrease to about 42% of controls by the third day after which intestinal dry weights increased gradually to about 63% of controls by the fifth day.

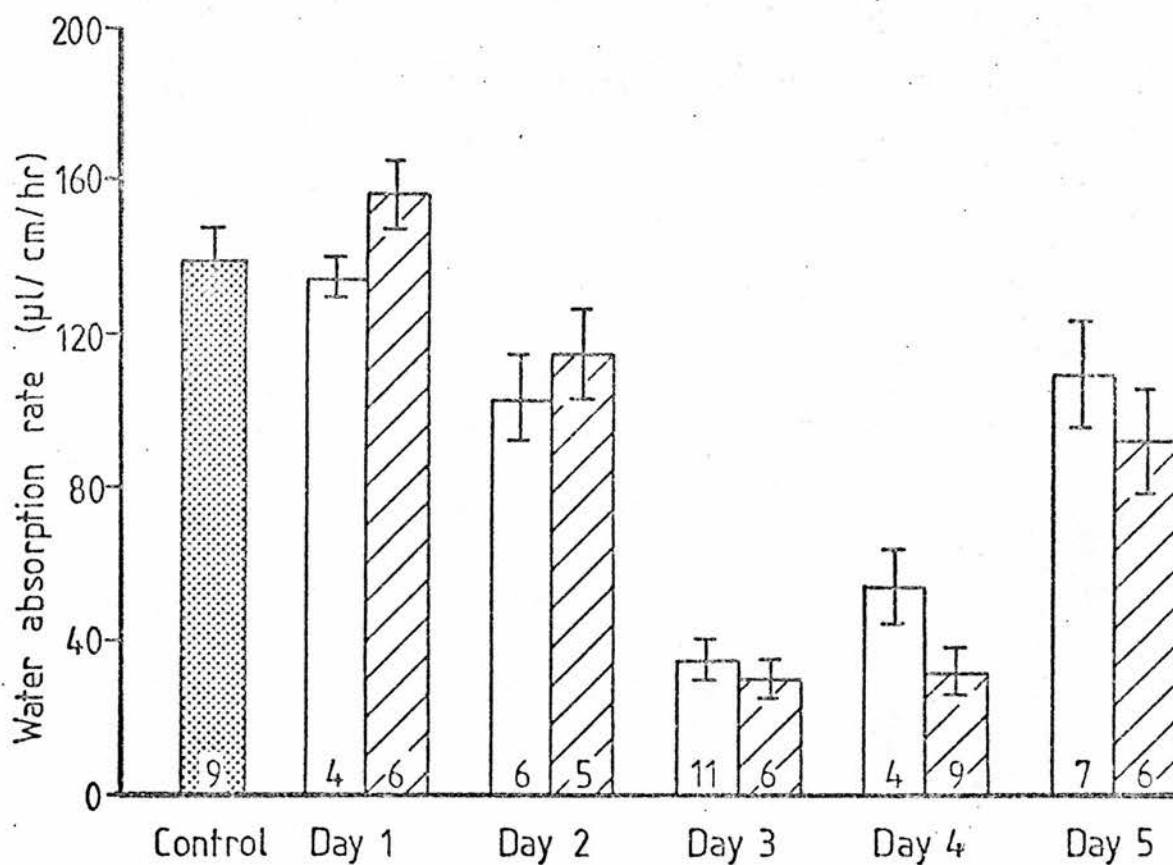


Fig. 8.3. Water absorption rates in jejunum plus ileum from male rats on each of the first 5 days following injection of the Tris salt (open bars) and the sodium salt (hatched bars) of 5-fluorouracil and in control rats (stippled bars). Values are mean \pm S.E.M. and the number of intestines is shown within the bars.

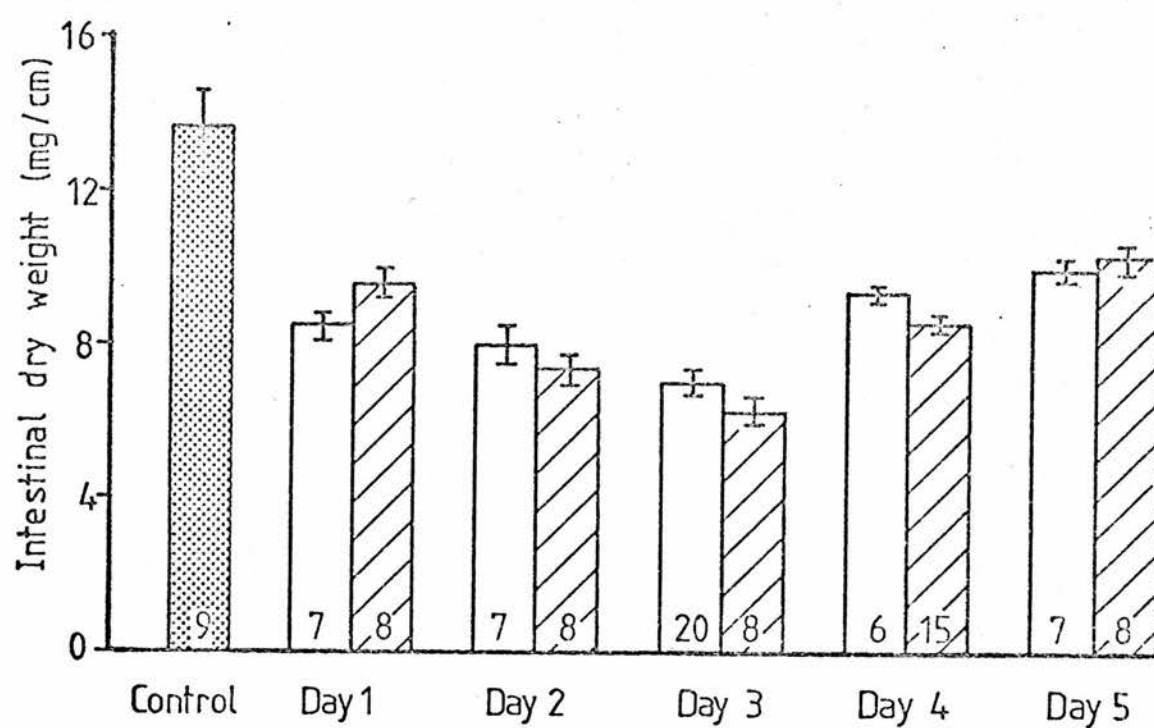


Fig. 8.4 Dry weights of jejunum plus ileum from male rats on each of the first 5 days following injection of the Tris salt (open bars) and sodium salt (hatched bars) of 5-fluorouracil and in control rats (stippled bars). Values are mean \pm S.E.M. and the number of intestines is shown within the bars.

Comparison of absorption rates from male and female rats

Figs. 8.5 and 8.6 show the intestinal water absorption rates and dry weights respectively from control, uninjected, rats and from rats 1 and 3 days after injection of either the Tris or the sodium salts of 5-fluorouracil. Both male and female rats were used and the results are shown separately. There was no difference between the intestinal water absorption rates of male and female control rats, although the dry weights of intestines from males were significantly greater than those from females ($P < 0.01$). Although both water absorption rates and dry weights on the third day after 5-fluorouracil administration were slightly greater in female rats than those in male rats, this difference was not significant ($P > 0.1$). Water absorption rates and dry weights were decreased to a similar extent after administration of either salt of 5-fluorouracil regardless of the sex of rat used.

Effects of ether anaesthesia and a Tris placebo injection on intestinal absorption and dry weight

5-Fluorouracil was administered under light ether anaesthesia. Since ether is known to stress rats (Martindale, 1977, p. 696; The Pharmaceutical Codex 1979, p. 337) and since the effects of ether anaesthesia and of Tris base on the intestine are not known, intestinal water absorption and dry weights were determined on the third day after exposure of female rats to temporary light ether anaesthesia alone or combined with an intraperitoneal injection of a matched Tris placebo. The results are shown in Table 8.2 and those from control female rats are included for comparison.

Neither ether anaesthesia or Tris placebo injection had any

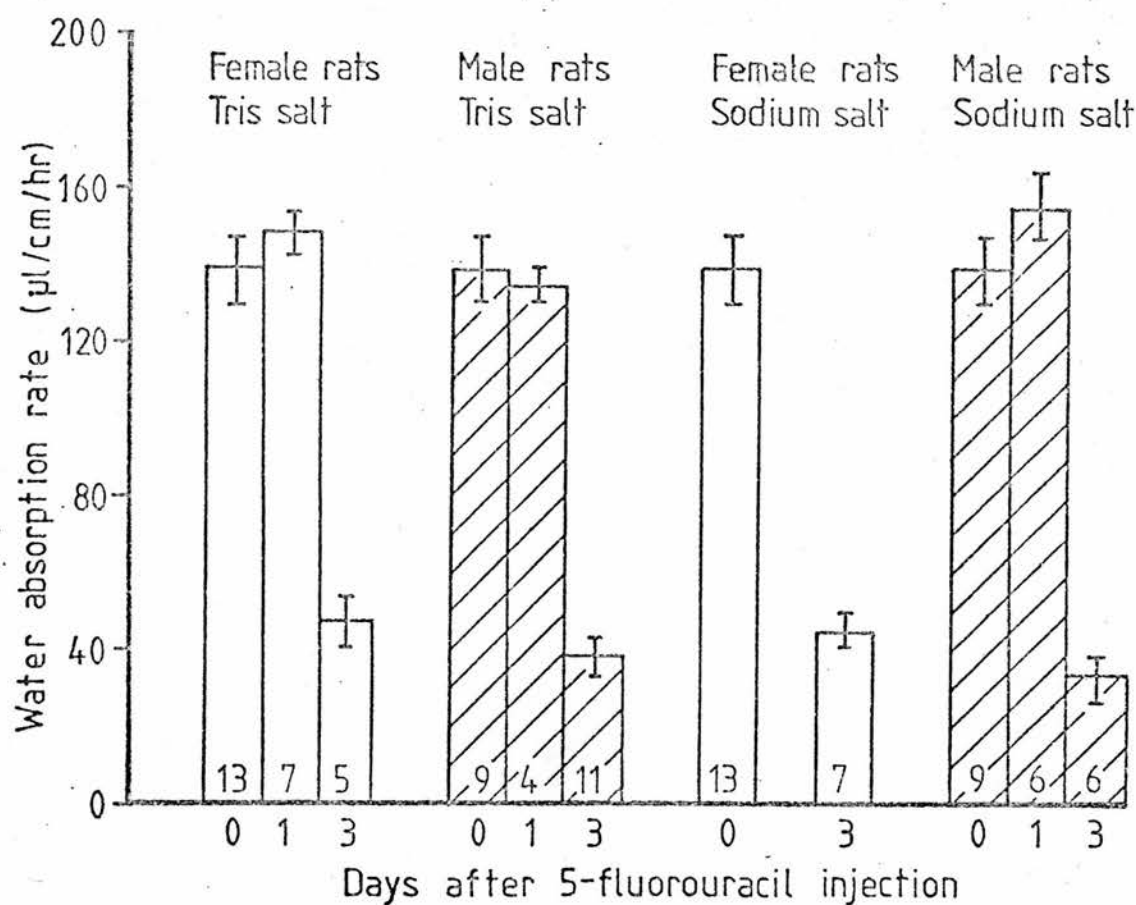


Fig. 8.5 Water absorption rates in jejunum plus ileum from male (hatched bars) and female (open bars) rats injected 1 or 3 days previously with either the Tris or sodium salt of 5-fluorouracil and from control rats (Day 0). Values are the mean \pm S.E.M. and the number of intestines is shown within the bars.

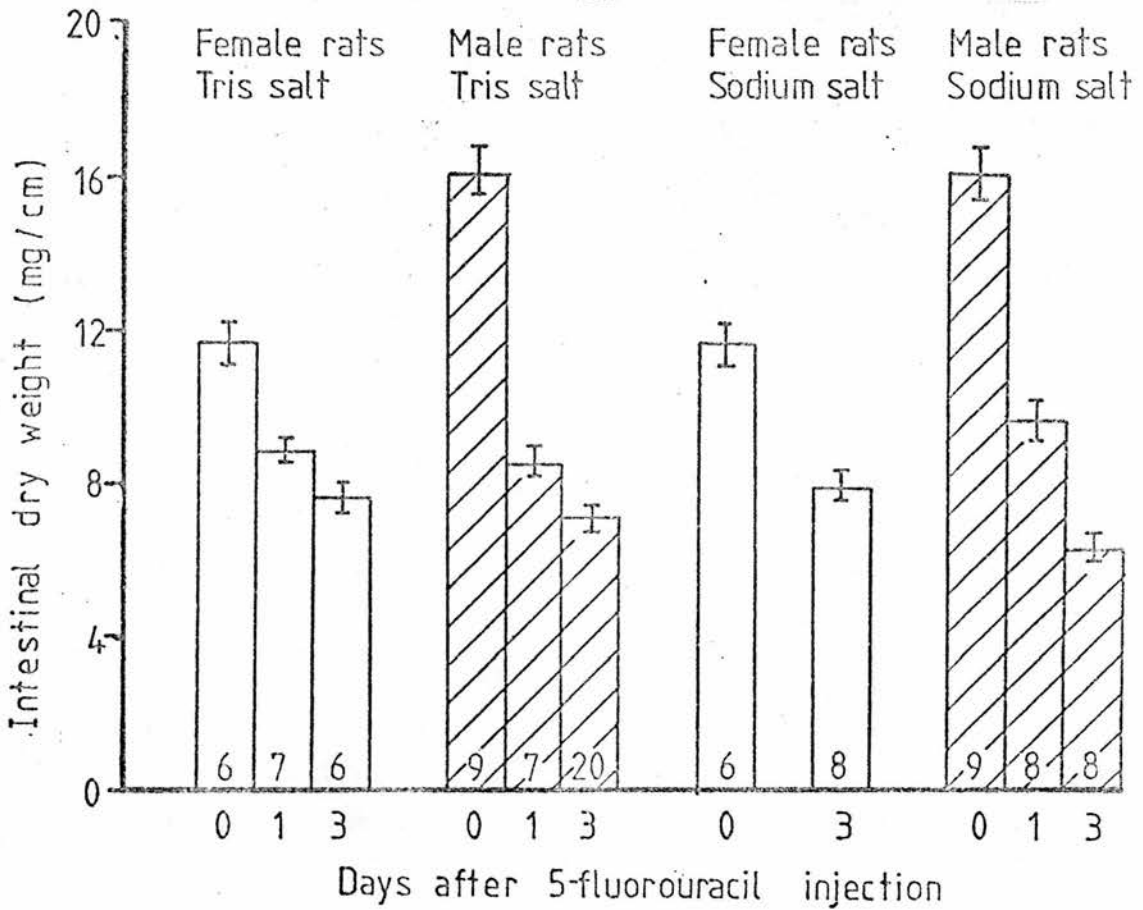


Fig. 8.6 Dry weights of jejunum plus ileum from male (hatched bars) and female (open bars) rats injected 1 or 3 days previously with either the Tris or sodium salt of 5-fluorouracil and from control rats (Day 0). Values are the mean \pm S.E.M. and the number of intestines is shown within the bars.

Table 8.2 Effect of temporary ether anaesthesia and of a Tris placebo injection on intestinal water absorption rates and dry weights 3 days after treatment

Stress	Water absorption rate ($\mu\text{l}/\text{cm}/\text{hr}$)	Intestinal dry weight (mg/cm)
None	138.69 ± 9.01 (13)	11.51 ± 0.37 (10)
Ether	143.34 ± 7.00 (6)	11.49 ± 0.41 (6)
Ether + Placebo	141.53 ± 5.32 (6)	11.34 ± 0.28 (6)

Values are the mean \pm S.E.M. and the number of intestines is shown in parentheses.

significant effect on intestinal water absorption rates or dry weights. Also injection of the Tris placebo had no effect on the food intake (Fig. 8.7), whereas injection of female rats with the Tris salt of 5-fluorouracil resulted in a marked decrease in the food intake similar to that seen in male rats (Fig. 8.1).

Effects of concentration and osmolarity of the Tris salt of 5-fluorouracil

The pH of the two preparation (sodium and Tris salts) of 5-fluorouracil is similar: 9.1 and 8.8 for the sodium and Tris salts respectively. However, the osmolarities of the two preparations differ (Table 8.3). That of the sodium salt (335 mOsm/l) is within the normal physiological range of plasma whereas that of the Tris salt is much higher (992 mOsm/l). Also, the concentration of the Tris salt (50 mg/ml, 384 mmol/l) is twice that of the sodium salt

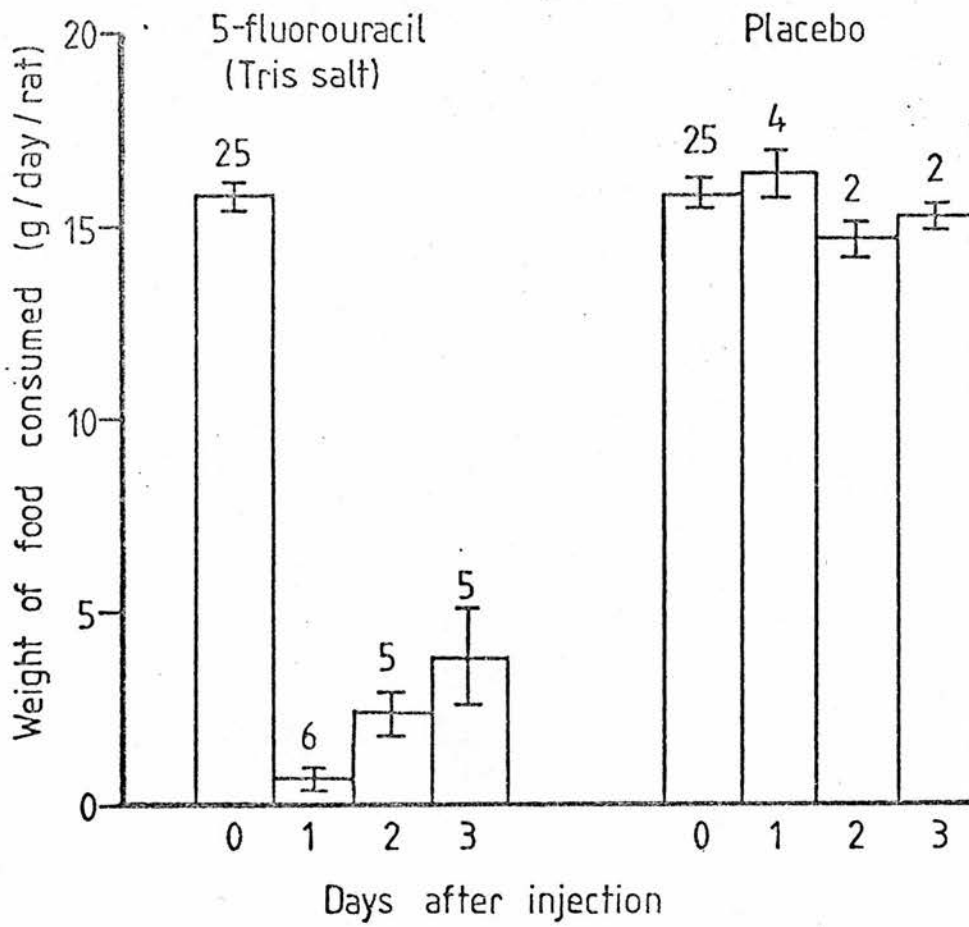


Fig. 8.7 Daily food intakes of female rats during the first 3 days after injection of the Tris salt of 5-fluorouracil or a Tris placebo. Values are the mean \pm S.E.M. and the number of observations on groups of 4 rats is shown above the bars.

Table 8.4a Effect of concentration and osmolarity of the Tris salt
of 5-fluorouracil on mortality, food intake and the incidence of
diarrhoea in male rats during the first 3 days after administration

<u>Injection</u>	<u>Mortality</u> (%)	<u>Incidence of</u> <u>diarrhoea (%)</u>	<u>Food intake</u> (g/rat/3 days)
5-fluorouracil	20.0 (70)	80.0 (20)	6.7 (5)
5-fluorouracil/ placebo 1:1	25.0 (8)	83.3 (6)	9.0 (1)
5-fluorouracil/ water 1:1	12.5 (8)	85.7 (7)	8.4 (1)

The total number of animals observed is shown in parentheses except for food intakes for which the number of observations on groups of 4 rats is shown in parentheses.

Table 8.4b Effect of concentration and osmolarity of the Tris salt
of 5-fluorouracil on intestinal water absorption and dry weights on
the third day after administration in male rats

<u>Injection</u>	<u>Water absorption rate</u> (μ l/cm/hr)	<u>Intestinal dry weight</u> (mg/cm)
5-fluorouracil	38.1 \pm 5.41 (12)	7.05 \pm 0.36 (20)
5-fluorouracil/ placebo 1:1	36.7 \pm 5.31 (5)	7.67 \pm 0.71 (6)
5-fluorouracil/ water 1:1	34.4 \pm 8.12 (4)	6.84 \pm 0.16 (7)

Values are means \pm S.E.M. and the number of intestines is shown in parentheses.

Table 8.3 Concentration, pH and osmolarity of the Tris and sodium salts of 5-fluorouracil

<u>Salt</u>	<u>Nominal Concentration* (mmol/l)</u>	<u>pH</u>	<u>Osmolarity (mOsm/l)</u>
Tris	384	8.8	992
Sodium	192	9.1	335

* Data supplied by Roche Products Ltd.

(25 mg/ml, 192 mmol/l). In order to determine whether either the osmolarity or the concentration of the Tris salt was responsible for the severe systemic toxicity associated with this salt, the effects of dilution of the Tris salt 1:1 with the matched Tris placebo (to give 5-fluorouracil concentration 25 mg/ml; osmolarity 1000 mOsm/l) and of dilution of the Tris salt 1:1 with distilled water (to give 5-fluorouracil concentration 25 mg/ml; osmolarity 500 mOsm/l) were determined.

Table 8.4a shows the mortality, food intakes and the incidence of diarrhoea during the first 3 days after administration of the Tris salt of 5-fluorouracil and after administration of the two dilutions of the Tris salt in male rats. Intestinal absorption rates and dry weights on the third day after injection are also shown in Table 8.4b. Neither of the two dilutions of the Tris salt had any significant effect on the severity of the toxicity observed after administration of the Tris salt of 5-fluorouracil, either in terms of the gross appearance of the rats or in terms of any of the measurements shown in Tables 8.4a and b. A significant mortality

was observed in all cases.

Effects of the dose of 5-fluorouracil on mortality and on intestinal absorption and dry weight

Fig. 8.8 shows the intestinal water absorption rates and dry weights estimated on the third day after injection of male rats with the Tris salt of 5-fluorouracil at various doses. Both water absorption rates and dry weights decreased in a similar manner with increasing doses of 5-fluorouracil; water absorption rates expressed per mg dry weight also decreased with increasing doses of 5-fluorouracil (Table 8.5). However, the ratio of the decrease in absorption rates and the decrease in intestinal dry weight is constant except for the lowest dose of 5-fluorouracil (Table 8.5).

Table 8.5 Effect of the dose of 5-fluorouracil on intestinal water absorption rates expressed per mg dry weight

Dose of 5-fluorouracil (mmol/kg body wt)	Water absorption rate (μ l/mg dry weight/h)	<u>Decrease in abs. rate</u> Decrease in dry weight (μ l/mg dry weight/hr)
1.44	5.24 \pm 0.70 (12)	11.1
1.08	6.01 \pm 1.34 (5)	11.4
0.72	7.44 \pm 0.70 (6)	11.3
0.36	9.46 \pm 1.68 (7)	9.4
Control	10.30 \pm 0.75 (9)	-

Values are the mean \pm S.E.M. and the number of intestines is shown in parentheses.

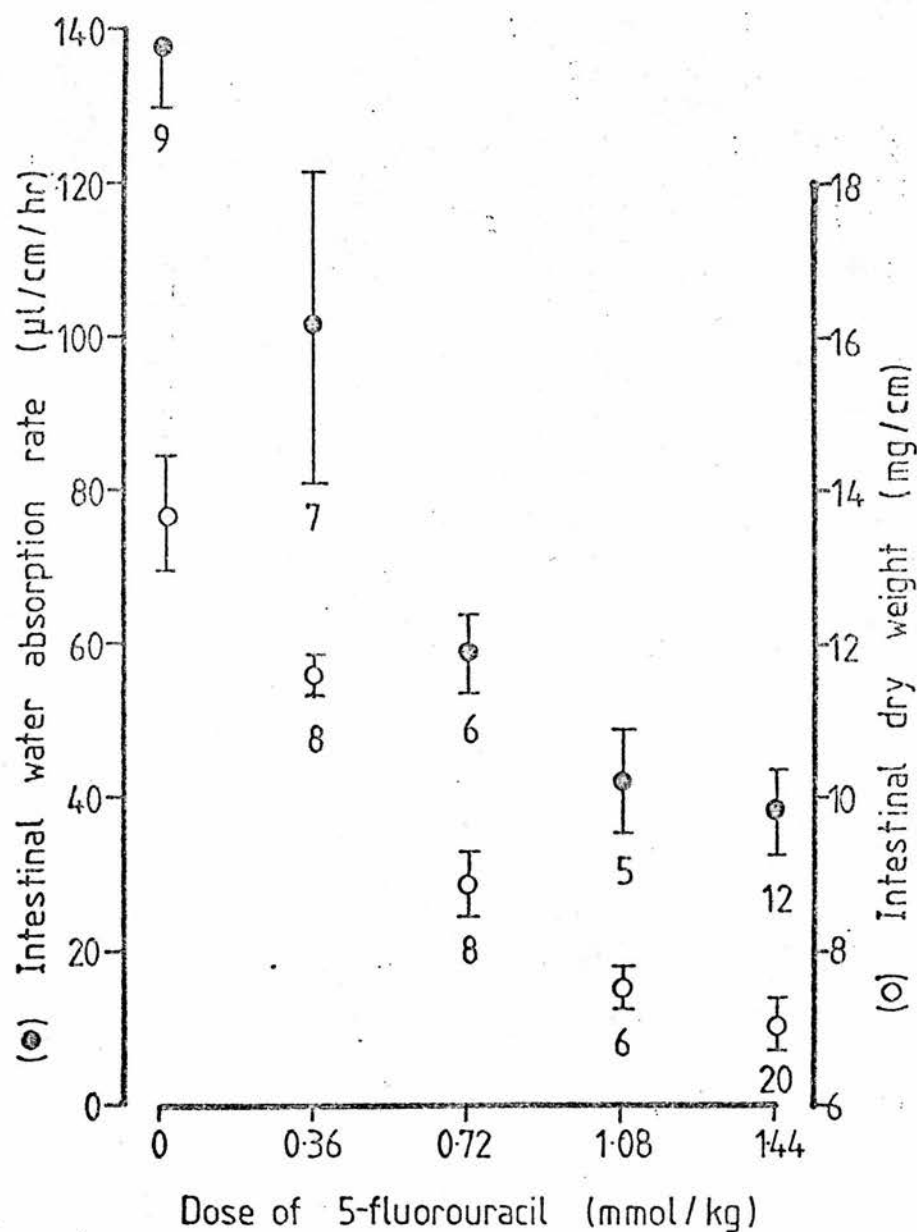


Fig. 8.8 Water absorption rates (●) and dry weights (○) of intestines from male rats 3 days after administration of various doses of the Tris salt of 5-fluorouracil. Values are the mean \pm S.E.M. and the number of intestines is shown below the points.

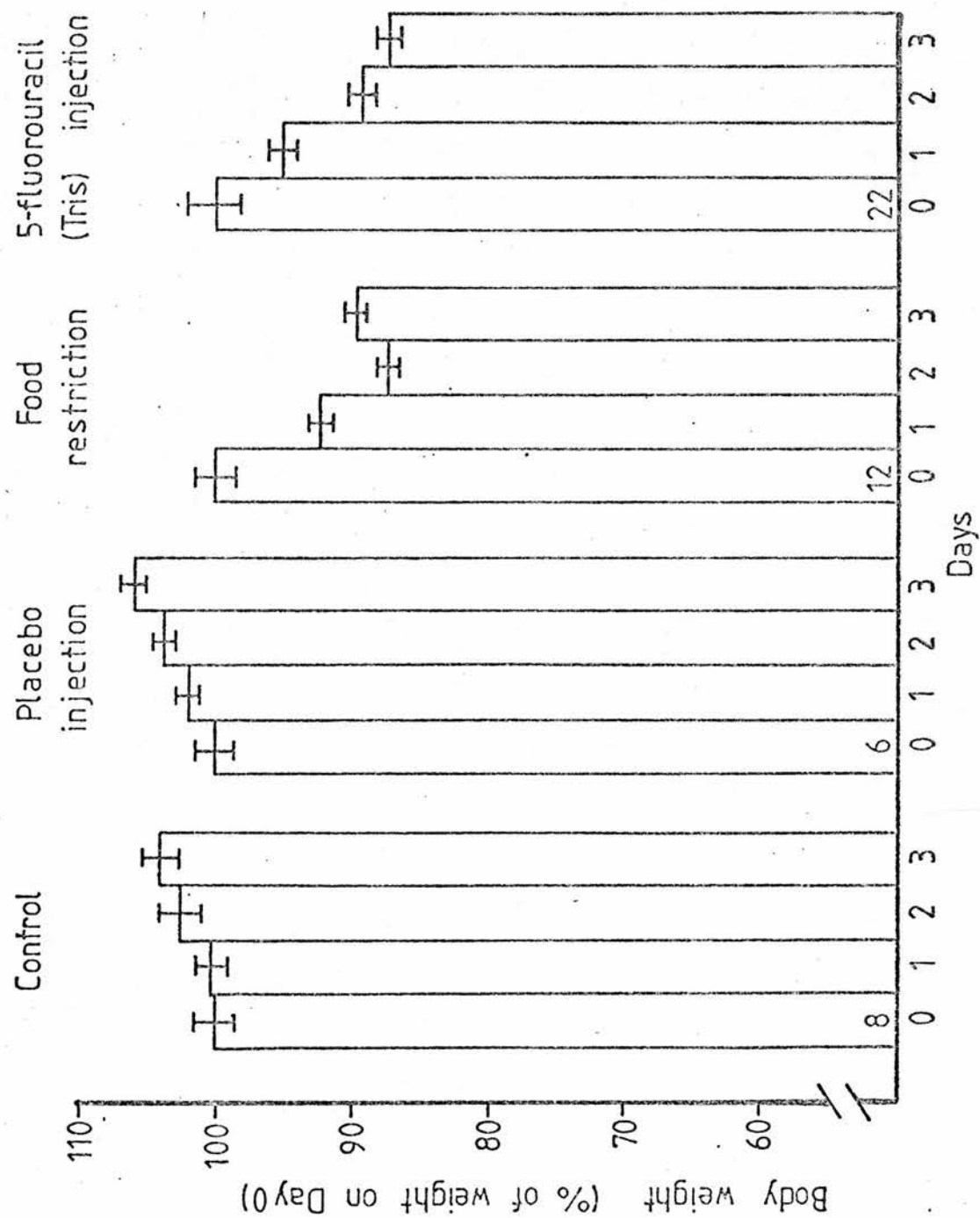
At all doses of 5-fluorouracil water absorption rates and dry weights were decreased significantly ($P < 0.02$, $P < 0.01$, $P < 0.01$, $P < 0.001$ for doses of 0.36, 0.72, 1.08 and 1.44 mmol/kg respectively) when compared with those from control, uninjected, rats. There were no deaths following administration of the two lower doses of 5-fluorouracil (0.36 and 0.72 mmol/kg). The mortalities at doses of 1.08 and 1.44 mmol/kg were similar, 18.2% (2 out of 11) and 21.4% (6 out of 28) respectively, and the slight difference between the absorption rates at these two doses was not significant ($P > 0.5$).

The gross appearance of the rats was consistent with these observations. At a dose of 1.08 mmol/kg the gross appearance of the rats was as described for rats injected with 5-fluorouracil at a dose of 1.44 mmol/kg. All 7 rats that survived the dose of 1.08 mmol/kg had diarrhoea by the third day as did 80% of the rats injected at a dose of 1.44 mmol/kg. None of the rats injected at the two lower doses of 5-fluorouracil (0.36 and 0.72 mmol/kg) developed diarrhoea.

Effects of 5-fluorouracil (Tris salt) and food restriction on body weight

Fig. 8.9 shows the body weights of female rats on each of the first 3 days of a food restriction regime designed to mimic the food intake after 5-fluorouracil administration and on each of the first 3 days after injection of the Tris salt of 5-fluorouracil. Those from control female rats and from female rats injected with the Tris placebo are included for comparison. The body weight decreased both after 5-fluorouracil injection and after food restriction. The decrease following food restriction was slightly greater over the

Fig. 8.9 Body weights of control female rats and on each of the first 3 days following Tris placebo injection, food restriction and 5-fluorouracil injection (Tris salt). Values are the means \pm S.E.M. of the weights expressed as a percentage of the body weight on the day of injection or the first day of food restriction (Day 0). Numbers of observations, each on a single animal, are shown within the bars for Day 0.



first two days than that after 5-fluorouracil treatment ($P < 0.02$). The increased food intake between the second and third day resulted in an increase in the body weight of the food restricted rats; in contrast, the body weight of the 5-fluorouracil-injected rats continued to decrease over this period.

The placebo and its associated temporary anaesthesia had no effect on the growth rate of the rats.

Effects of 5-fluorouracil (Tris and sodium salts) and food restriction on mucosal DNA content

The mucosal DNA content of female rats on the first and third days of a food restriction regime and on the first and third days after injection of the Tris or sodium salt of 5-fluorouracil is shown in Fig. 8.10. The mucosal DNA content had decreased to 47% of the normal content on the first day after administration of the Tris or sodium salt of 5-fluorouracil; in contrast, food deprivation for 1 day had no effect on the mucosal DNA content. By the third day after 5-fluorouracil injection the DNA content had decreased to 38% of the control value; food restriction for three days resulted in a much smaller, but significant ($P < 0.02$), decrease in the mucosal DNA content to 86% of the control value.

Effects of 5-fluorouracil (Tris salt) and food restriction on water absorption

The rates of water absorption after injection of 5-fluorouracil or after food restriction were determined: these rates have been expressed relative to (a) total jejunum plus ileum, (b) unit length of intestine, (c) unit dry weight of whole small intestine, and (d)

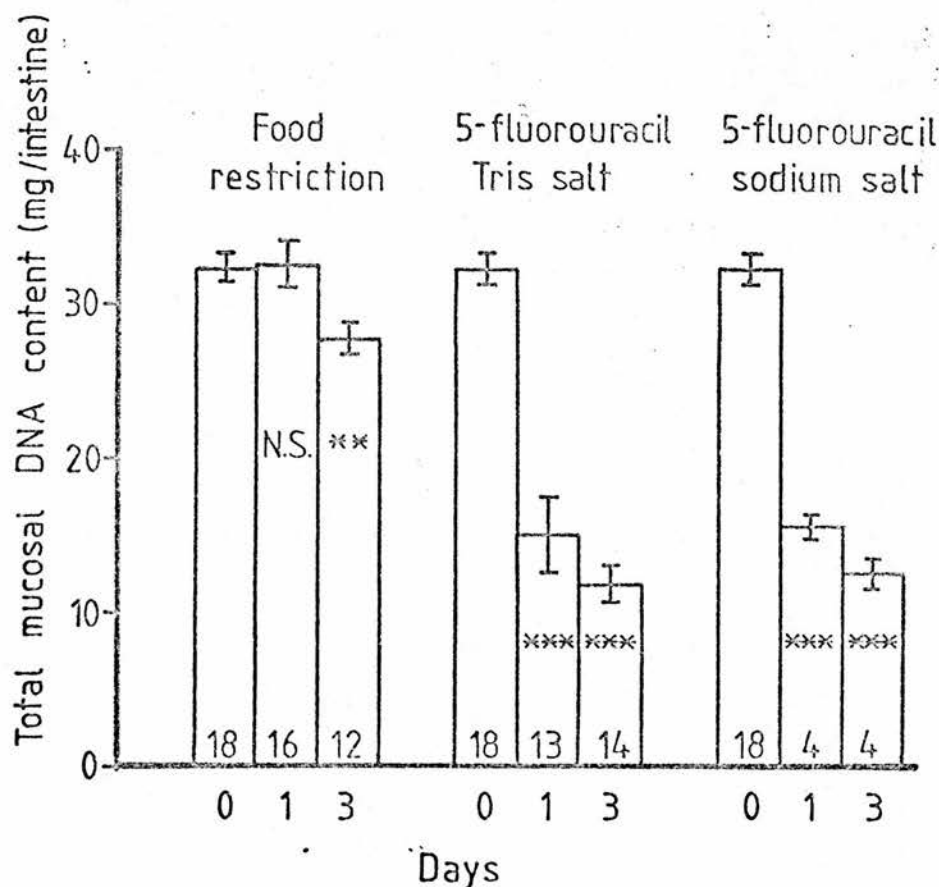


Fig. 8.10 DNA content of the mucosa from the whole jejunum plus ileum from control female rats, and on the first and third days of the food restriction regime and following administration of either the Tris or sodium salt of 5-fluorouracil. Values are the mean \pm S.E.M. and the numbers of intestines are shown within the bars. The significance levels of the differences from values in control rats are indicated thus: *** $P \leq 0.001$, ** $P \leq 0.02$, N.S. $P \geq 0.05$.

mucosal DNA content (Figs. 8.11 and 8.12). There was no significant change in water absorption rates expressed per whole small intestine one day after 5-fluorouracil treatment; by the third day after 5-fluorouracil injection they had decreased to 30% of control values. On the other hand, when absorption rates were expressed relative to total intestinal dry weight or to the mucosal DNA content, a dramatic increase was observed after one day and by the third day the absorption rates had decreased to 70 - 80% of control values (Fig. 8.11).

After food deprivation for one or three days the water absorption rate expressed per whole small intestine was decreased to approximately 70% of the control rate (Fig. 8.12). The decreased absorption rate was not quite significant when any of the other three reference systems were used.

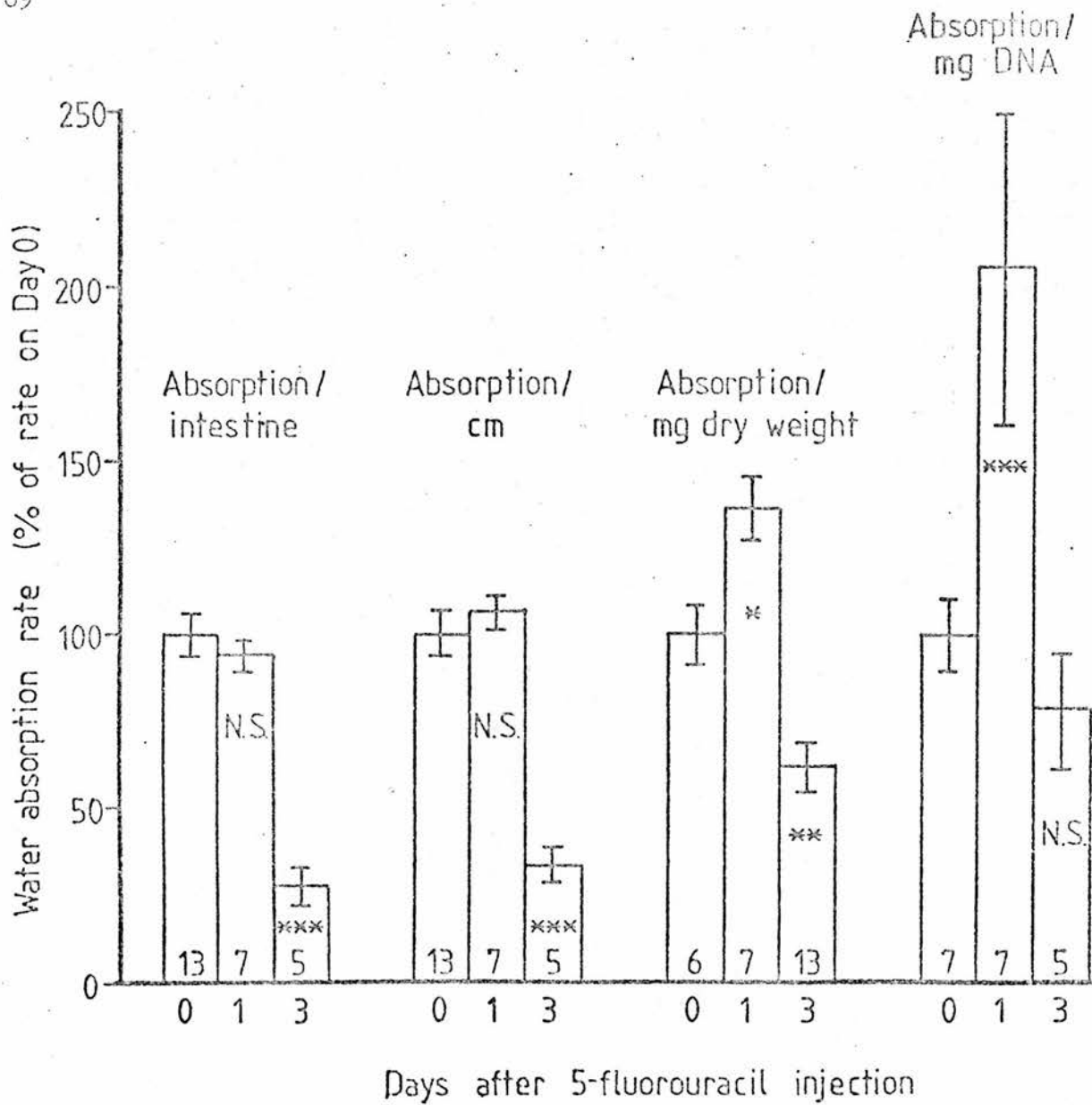


Fig. 8.11 Water absorption rates in jejunum plus ileum from female control rats and from female rats which had received 5-fluorouracil (Tris salt) 1 or 3 days previously. Rates are expressed as a percentage of control (Day 0) rates using four different reference systems: whole small intestine, cm length of intestine, mg dry weight of intestine and mg mucosal DNA content. Values are means \pm S.E.M. and the numbers of intestines are shown within the bars. The significance levels of the differences from the values in control rats are indicated thus: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.02$, N.S. $P \geq 0.05$.

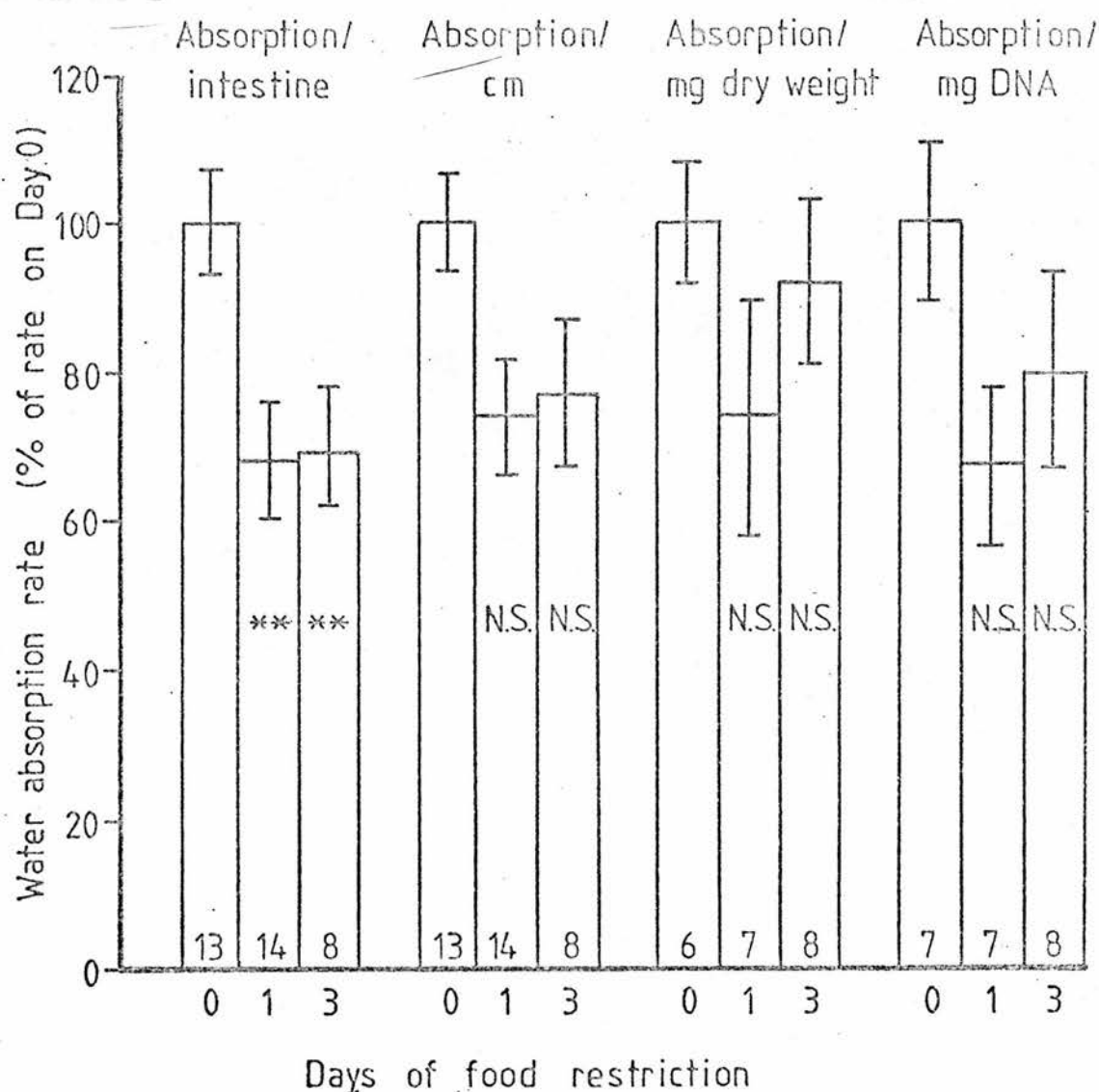


Fig 8.12 Water absorption rates in jejunum plus ileum from control female rats and from female rats following food deprivation for 1 day or food restriction for 3 days. Rates are expressed as a percentage of control rates using each of four different reference systems: whole small intestine, cm length of intestine, mg dry weight of intestine and mg mucosal DNA content. Values are means \pm S.E.M. and the numbers of intestines are shown within the bars. The significance levels of the differences from the value in control rats are indicated thus: ** $P < 0.02$, N.S. $P \geq 0.05$.

DISCUSSION

This chapter describes the differential effects of 5-fluorouracil and starvation on the intestine. During this study the suppliers of the 5-fluorouracil changed the salt form of the drug supplied and this resulted in the totally unexpected observation of the differential toxicities of the Tris and sodium salts of 5-fluorouracil. It soon became clear that the two salts have very different effects on the food intakes of rats but the intestinal toxicities of the two salts are very similar. It is, therefore, appropriate to discuss the differential toxicities of the Tris and sodium salts of 5-fluorouracil in this chapter since the two salts offer a further means of dissociating the effects of 5-fluorouracil per se from those of the reduced food intake. Thus the first part of this discussion is concerned with the intestinal toxicity of 5-fluorouracil and includes a discussion of the differences in the systemic toxicities of the two salts of 5-fluorouracil. The effects of 5-fluorouracil on the intestine are then compared with those of starvation. Finally, the effects of the choice of reference system for the expression of intestinal absorption are discussed.

Intestinal toxicity of 5-fluorouracil

The most striking (and novel) observation reported in this chapter was that of the differential toxicities of the two salts of 5-fluorouracil. The difference was clear simply from the appearance of the rats a few hours after administration of 5-fluorouracil. All those injected with the Tris salt of 5-fluorouracil became very cold and lethargic and, out of a total of 319 rats injected, 48 rats died.

In contrast, administration of the sodium salt of 5-fluorouracil to rats was never associated with any of these signs of systemic toxicity and no deaths were recorded after injection of 449 rats. Since there was no obvious explanation for the marked difference in the systemic toxicities of the two salts the effects of the Tris and the sodium salts of 5-fluorouracil on the rat were studied in detail.

The difference in the systemic toxicities of the two salts was also apparent in the effects on food intakes and body weights. The Tris and sodium salts of 5-fluorouracil had very different effects on the food intakes of rats (Fig. 8.1). During the first 24 hours after administration of the Tris salt of 5-fluorouracil the food intake decreased to only 7% of the normal daily intake. The food intake gradually increased over the next few days such that by the fifth day the food intake was only 24% of the normal daily intake. In contrast, during the first 24 hours after administration of the sodium salt of 5-fluorouracil, the food intake decreased only to 50% of the normal daily intake. The food intake decreased further to only 7% of the normal intake by the third day but rapidly increased to 55% by the fifth day. The total food intake during the first 5 days after administration of the sodium salt (34% of the normal intake over the same period) was twice that after administration of the Tris salt of 5-fluorouracil, (16% of the normal intake).

These different effects on food intakes were reflected by the changes in body weight following 5-fluorouracil administration. During the first four days after administration of 5-fluorouracil the decreased in body weight was consistently significantly greater

after administration of the Tris salt than after administration of the sodium salt of the drug ($P < 0.02$). The increase in food intake on the fifth day after administration of the sodium salt of 5-fluorouracil resulted in an increase in the body weight of the rats, whereas the body weights of rats injected with the Tris salt continued to decrease (Fig. 8.2).

Although these marked differences were observed in the systemic toxicities and the effects on food intakes and body weights, the intestinal toxicities of the two salts of 5-fluorouracil were similar (Figs. 8.3 and 8.4). There was no significant difference between either water absorption rates or intestinal dry weights on each of the first 5 days after administration of the Tris and sodium salts of 5-fluorouracil (Figs. 8.3 and 8.4 respectively). Water absorption rates were not affected on the first day after 5-fluorouracil administration. Severe impairment of water absorption to 26% of that in control, uninjected, rats was observed by the third day after injection of either salt of 5-fluorouracil. By the fifth day absorption rates had increased to about 70% of the control rate. These observations differ from those of Gardner *et al* (1978). They reported increased rates of water and glucose absorption, to 150% and 120% of control rates respectively, on the first day after administration of the Tris salt of 5-fluorouracil in female rats. Also, glucose and water absorption rates were decreased to about 40% of the control rate by the third day but showed no recovery on the fifth day, as observed in this study. However, the control rate for water absorption reported by Gardner *et al* (1978) of 131.5 ± 5.75 $\mu\text{l}/\text{cm}/\text{hr}$ agrees well with the control rate observed in the present study (138.7 ± 9.0 $\mu\text{l}/\text{cm}/\text{hr}$). These differences cannot be explained

but it is of note that Roche et al (1970) did not observe any effect on glucose absorption in vivo in rats on the first day after 5-fluorouracil administration. Thus their results are consistent with those reported here, and not with those of Gardner et al (1978).

Although there was no effect on water absorption on the first day after 5-fluorouracil administration, both the intestinal dry weight and the mucosal DNA content had decreased to about 50% of the normal values (Figs. 8.4 and 8.10). Therefore, if the water absorption rates were expressed relative to dry weight or to the mucosal DNA content, a dramatic increase would be observed on the first day after 5-fluorouracil administration. This observation is discussed further below.

The differential toxicities of the Tris and sodium salts of 5-fluorouracil

The actual cause of the severe systemic toxicity associated with the Tris salt of 5-fluorouracil is not known. Tris base has been reported to be toxic to some tissues (Robertson, 1970). However, no toxic symptoms were observed following ether anaesthesia and injection of a matched Tris placebo (Table 8.2). Also, the toxicity does not appear to be associated with the higher concentration and osmolarity of the Tris salt of 5-fluorouracil since reduction of both by a factor of two had no significant effect on the severity of the toxic symptoms or on the mortality (Table 8.4a).

In Chapter 9 a difference in the pharmacokinetics of the two salts of 5-fluorouracil is reported. Higher plasma levels of 5-fluorouracil are obtained after intraperitoneal injection of the Tris salt than after injection of the sodium salt at the same molar

dose. The higher plasma levels of 5-fluorouracil might explain the differential toxicities of the two salts but, as discussed in Chapter 9, this is thought to be unlikely. It is concluded that the difference in the pharmacokinetics is evidence of a fundamental difference in the properties of the Tris and sodium salts of 5-fluorouracil and that it is probably a property of the Tris salt of 5-fluorouracil per se that is responsible for the severe systemic toxicity and mortality associated with its use.

Although the cause of death is not known, severe pancreatic damage was observed on the third day after injection of the Tris salt of 5-fluorouracil but not after injection of the sodium salt of 5-fluorouracil. Similarly, Martin et al (1969) reported pancreatic damage in rats after three consecutive days of 5-fluorouracil administration.* They reported an accumulation of zymogen granules in the pancreas of 5-fluorouracil-treated rats and suggested that 5-fluorouracil inhibits the normal secretory mechanism of the pancreas. Although it is not known how soon after 5-fluorouracil administration pancreatic damage is apparent, Liss and Chadwick (1974) reported extensive accumulation of 5-fluorouracil-2-C¹⁴ in tissues of rats and mice 2 hours after an intravenous injection and these included the pancreas. Therefore pancreatic damage might be partly responsible for the toxic symptoms observed 3 to 4 hours after an intraperitoneal injection of the Tris salt of 5-fluorouracil. Acute pancreatitis is a potential cause of death.

*Assumed to be the Tris salt of 5-fluorouracil since it must have been supplied during the period in which only the Tris salt was available from Roche in the U.K.

Intestinal absorption in male and female rats

Fisher (1955) reported a difference in the intestinal absorption rates of both glucose and water between male and female rats. The mean intestinal water absorption rate in female rats was 236 $\mu\text{l}/\text{cm}/\text{hr}$ compared with 188 $\mu\text{l}/\text{cm}/\text{hr}$ in male rats. Both of these rates, estimated by the re-circulation perfusion technique of Fisher and Parsons (1949), were higher than those of Gardner *et al* (1978) and those estimated in the present study by the method of Fisher and Gardner (1974); the difference is probably due to the use of different techniques and strain of rats.

In this study no difference was observed between the intestinal water absorption rates estimated in male and female rats. This was true for animals fed on a standard diet (Fig. 8.5) or on an 'elemental' diet (Chapter 10, Table 10.9). Although the dry weights of intestines from male rats were 38% greater than those from female rats, the mucosal DNA contents did not differ (females - 32.0 mg/intestine; males - 35.5 mg/intestine reported in Chapter 11). This suggests that the higher intestinal dry weight observed in male rats is not due to a greater number of intestinal epithelial cells, but may be due to a greater size of the cells or a greater thickness of the muscle layer.

The intestinal toxicity of 5-fluorouracil is similar in male and female rats. There is no significant difference in the intestinal absorption rate on the third day after administration of either salt of 5-fluorouracil in male and female rats (Fig. 8.5). Intestinal dry weights are also similar on the third day after 5-fluorouracil administration in male and female rats (Fig. 8.6). Since the dry

weight of intestines from male rats is greater than that of female rats, the decrease in dry weight was greater in male rats (56%) than in female rats (35%). If the intestinal epithelial cells of male rats are larger than those of female rats, or if the muscle layer is larger, this difference may not exclude the loss of a similar number of cells from the intestinal epithelium of the two sexes.

The effects of 5-fluorouracil on food intakes and body weights of male and female rats were also similar (Figs. 8.1 and 8.7 and Figs. 8.2 and 8.9 respectively). However, the daily food intakes and growth rates of male rats were 20% and 146% greater respectively than those of female rats (see Chapter 10, Table 10.7), and therefore direct comparisons of these parameters should not be made between the two sexes.

Differential effects of 5-fluorouracil and starvation on the intestine

The results presented in this chapter show clearly several fundamental differences between the changes in absorption rates following 5-fluorouracil injection and those after the food restriction regime designed to mimic the animals' food intake after 5-fluorouracil injection.

Water absorption by the whole small intestine is greatly decreased after 5-fluorouracil injection (Fig. 8.11). However, the reduced food intake by itself results in a much smaller, although significant ($P < 0.02$), decrease in water absorption (Fig. 8.12). Hence the effects of 5-fluorouracil on absorption cannot wholly be attributed to food restriction. This result agrees with Levins' (1968) observation of decreased absorption of glucose, galactose and

fructose in fasted rats after injection of 5-fluorouracil when compared with fasted controls - but see below. Also, the use of both the Tris and sodium salts of 5-fluorouracil, which have different effects on the food intake pattern after injection, has demonstrated further the difference between the effects of 5-fluorouracil on absorption and the effects of the consequent reduced food intake on absorption. The intestinal toxicity of the two salts is similar (Figs. 8.3 and 8.4) and they have identical effects on the mucosal DNA content (Fig. 8.10) although the overall food intake during the first 3 days after injection of the sodium salt of 5-fluorouracil is three times that after injection of the Tris salt of 5-fluorouracil.

Bounous and Maestracci (1976) reported a greater reduction in intestinal sucrase and leucyl-naphthylamide-hydrolysing activities in rats following 5-fluorouracil administration than was observed when the daily food intake of rats was restricted to that observed during 5-fluorouracil treatment. They also reported that body weight losses after 5-fluorouracil (sodium salt) administration were greater than those observed after the reduced food intake. In contrast, body weight losses after food restriction based on the food intakes observed after administration of the Tris salt of 5-fluorouracil, were greater on the first two days than after administration of the Tris salt of 5-fluorouracil (Fig. 8.9). This is consistent with the observations, reported in this chapter, that the food intakes after injection of the sodium salt were greater than those after injection of the Tris salt of 5-fluorouracil. Hence the food restriction regime for the Tris salt allowed less food than would that for the sodium salt of 5-fluorouracil.

As noted in the introduction to this chapter, the food restriction regime could not mimic exactly the feeding pattern of rats after 5-fluorouracil injection, but did mimic the total daily intake. The regime adopted differs slightly from the food intakes shown in Fig. 8.7, since this figure includes all observations on food intakes whereas the food restriction regime was based only on a preliminary experiment. The daily food intake can be controlled, but the hour-to-hour food intake of the rats is not easy to control. For example, when the food intake of rats was restricted all the food (5.4 g) presented on the second day was eaten immediately, thus leaving a fast of at least 12 hours before perfusion on the third day. However, this reservation does not apply to the first day after injection of the Tris salt of 5-fluorouracil since the food intake then was negligible. A 24 hour fast has considerable effects on absorption; water absorption by the whole small intestine is decreased by 32% of control values (Fig. 8.12). This indicates the potential hazard in the practice of preparing animals for absorption studies by an overnight fast as is routinely performed in many laboratories (Heller, 1954; Steiner and Gray, 1969; Cheng et al, 1971) especially since this, for a nocturnal feeder (see Chapter 11), is equivalent to a 24 hour fast. This is claimed to aid removal of the intestinal lumen contents prior to use of the intestine. However, experience from this study suggested that the 24 hour fast did not completely clear the intestine of solid debris and there thus seems to be little merit in this practice.

Effects of 5-fluorouracil on cell numbers and cell function

A change in absorption rates could be a result of either a change in the number of absorbing cells or of a change in the

absorbing capacity of individual cells. Thus the decreased absorption rates following 5-fluorouracil administration could be a direct result of a loss of cells from the intestinal mucosa or a result of damage to existing absorbing cells such that their function is impaired or, it may result from both of these causes. Distinction between these two causes of impaired absorption is important since in the first case recovery of absorption is dependent upon production of new cells, whereas in the latter case recovery may also occur via repair of damaged cells. Batt and Peters (1976) have suggested that absorption rates should be expressed per enterocyte, estimated by mucosal DNA measurement, in order to differentiate between these two possibilities. Although there was no effect on the absorption rate by the whole small intestine on the first day after 5-fluorouracil injection, the absorption rate expressed per mg DNA is doubled (Fig. 8.11). Roche *et al* (1970) also reported a change in cell numbers with no change in absorption rates, expressed per unit length of small intestine, 1 day after 5-fluorouracil injection. Since 5-fluorouracil inhibits DNA synthesis and therefore prevents crypt cell division in the intestinal epithelium, the 54% decrease in the mucosal DNA content 1 day after injection probably represents a reduced number of crypt and immature villus cells (i.e. non-absorbing cells) with, at this stage, little or no change in the number of mature absorbing cells at the tip of the villi. Furthermore, the mucosal DNA content also includes cells of the lamina propria. Thus, since DNA is not a specific index of the number of absorbing cells, it is not possible to use it in a study of the effects of an antimetabolite on a heterogeneous population of cells to determine whether the absorptive capacity of individual absorbing cells has been altered.

Levin (1968) suggested that changes in absorption rates after 5-fluorouracil treatment were due to changes in cell numbers since absorption rates expressed per unit dry weight of intestine were not affected on the third day of three consecutive daily injections. However, Levin (1968) starved the rats during 5-fluorouracil treatment and compared the results with those from fasted controls. The severity of the intestinal toxicity of 5-fluorouracil is increased by a 24 hour fast before administration (see Chapter 11). This may explain why the results of Levin (1968) differ from those observed in this study; water absorption rates expressed per unit dry weight of intestine were significantly less on the third day after 5-fluorouracil than the control rate and less than that on the third day of food restriction. This result contrasts with that of Levin (1968) but this does not unequivocally indicate that the decreased absorption rate on the third day after 5-fluorouracil injection is a result of a decrease in the absorptive capacity of individual cells as well as a decrease in the number of absorbing cells. The reservations already discussed with respect to DNA as a reference basis also apply to the use of dry weight and unfortunately prevent the solution of this problem.

The effects of varying the dose of 5-fluorouracil suggested that the reduced water absorption rate seen on the third day after 5-fluorouracil injection might be due to a decrease in cell numbers. The pattern of change in intestinal absorption rates and dry weights is similar and there is a constant loss of absorptive capacity per unit loss of dry weight (Fig. 8.8 and Table 8.5).

Thus, while it is not possible to conclude that only the number of absorbing cells has been changed by 5-fluorouracil, and to exclude

the possibility that the absorptive capacity of individual cells has also been reduced, it can be noted that the results are perfectly consistent with this hypothesis. Further, the parallel changes seen in Fig. 8.8 lend some support, though tentative, to it. Hence, although Levin's actual observations (discussed above) differ from those reported here, it seems likely that his conclusion was correct.

The effects of different reference systems

Water absorption rates have been expressed with respect to four different reference systems: whole small intestine, unit length of intestine, unit dry weight of intestine and mucosal DNA content. The results illustrate clearly how the choice of reference system affects both the quantitative and qualitative pattern of results. Whilst absorption rates are not affected on the first day after 5-fluorouracil administration when referred to whole small intestine or per unit length of intestine, there is a significant increase in absorption rates referred to unit dry weight or mucosal DNA content (Fig. 8.11). On the third day after 5-fluorouracil administration the absorption rates are decreased significantly except when they are referred to the mucosal DNA content. The effect of the reference system on changes in absorption rates following the food restriction regime is less dramatic. However, whilst there is a significant decrease in absorption by the whole small intestine, none of the other three reference systems reflect this effect (Fig. 8.12).

Levin et.al (1965) showed that the direction of change of enzyme activities as a result of starvation can even be reversed by use of a different reference system. The total peptide hydrolase activity against L-Leu-Gly was decreased on an absolute basis in rats after a 3 day fast but there was an increase when the activity was referred

to unit wet weight of intestine. Food restriction or starvation can cause a decrease in absorption rates when referred to the whole or a specific length of the small intestine or, in contrast, an increase when expressed on a weight basis (Kershaw et al, 1960; Levin, 1970; Lis et al, 1972; Newey et al, 1970). Newey et al (1970) and Lis et al (1972) have discussed the possibility of obtaining misleading results when absorption rates are expressed on a weight basis. Newey et al (1970) point out that although the absorption rate referred to intestinal weight is increased in fasted animals this is of no importance to the rat in vivo, since the total weight of the intestine is decreased. Lis et al (1972) stress the difficulty in comparing results from studies of the effects of starvation on intestinal absorption due to the use of different reference systems. They conclude that since the length of the small intestine is not affected by starvation, it is a more suitable reference basis than intestinal weight. The results presented in this chapter agree with this conclusion since both food restriction and 5-fluorouracil injection affect the intestinal dry weight and mucosal DNA content and changes in absorption referred to these two reference systems do not reflect the changes in the total absorptive capacity of the small intestine. Absorption per whole small intestine is a useful index, especially when seeking the overall effect on physiological function. Nevertheless, Gardner and Heading (1979) reported that the length of the intestine was reduced by 5-fluorouracil. Although this effect was not confirmed in this study the observation of Gardner and Heading (1979) detracts from the value of expressing absorption rates in terms of unit length.

CHAPTER 9

Plasma pharmacokinetics of the Tris and sodium salts of 5-fluorouracil in the rat

Introduction

The differential toxicities of the Tris and sodium salts of 5-fluorouracil were described in the previous chapter (Chapter 8). It became clear that administration of the Tris salt of 5-fluorouracil was associated (particularly) with an obvious general malaise which was seen in all rats, significant mortality (16%) and pancreatic lesions. In contrast, no such toxicity was associated with the sodium salt of 5-fluorouracil.

There are many examples of differences in the bioavailability* and therapeutic response between various commercial preparations of the same drug (Bowman and Rand, 1980), but such a marked difference in the systemic toxicities of two forms of the same drug is uncommon. Although there are well defined guidelines to the pharmaceutical industry for testing new medicinal products (e.g. A.B.P.I. 1977), the differential toxicities of the two salts of 5-fluorouracil appear not to have been detected. It was, therefore, of great interest to determine the nature of the difference in the systemic toxicities of the two salts.

* The bioavailability of a drug refers to that quantity of the administered dose which arrives in a suitable form and concentration at the sites within the body where it will exert its biological effect.

A difference in the bioavailability or simply in the rate of absorption of the Tris and sodium salts of 5-fluorouracil might explain the different systemic toxicities observed following administration of each salt. Therefore a method was developed to study the plasma pharmacokinetics of the two salts of 5-fluorouracil in the rat.

Blood sampling

The removal of a number of blood samples from the conscious rat presents a major problem because of the lack of accessible blood vessels. 'Tail-snips' are commonly used to obtain small blood samples but this method is unsuited to pharmacokinetic studies since the bleeding cannot be controlled and there is no opportunity for fluid replacement. A significant change in the blood volume is undesirable since it will interfere with the pharmacokinetics. Therefore blood sampling was carried out on the anaesthetised rat via a cannulated blood vessel. Although anaesthesia may affect the pharmacokinetics of 5-fluorouracil, the studies were simply comparative between the two salts, and so this was not considered to be an overriding disadvantage. The carotid artery was chosen for cannulation because it is a large vessel (about 0.5 mm diameter), is easily located and is able to withstand reasonably rough handling. An artery is preferable to a vein since the arterial pressure aids blood sampling and since veins tend to collapse around the cannula. The withdrawn fluid was replaced immediately with heparinized saline both to keep the cannula patent and to maintain the blood volume.

The preparation was viable for around 4 hours. However, after injection of the Tris salt of 5-fluorouracil, mortality was high

during the third and fourth hours and few animals survived the 4 hour experimental period; after injection of the sodium salt of 5-fluorouracil most animals survived for at least 200 minutes. This mortality was presumably due to a combination of the toxic effects of 5-fluorouracil, anaesthesia and surgery. However, the deaths did not appear to interfere with the pharmacokinetic studies; in retrospect, the plasma levels of 5-fluorouracil were similar in rats that died and in those that survived (see Fig. 9.6).

Estimation of plasma 5-fluorouracil

There are several methods available for determination of 5-fluorouracil at low concentrations (ng/ml) in plasma. However, these methods are complex and most involve the use of high pressure liquid chromatography or gas-liquid chromatography. These two techniques were not available and are not easily developed. The high pressure liquid chromatography technique used in the University Department of Clinical Pharmacology in the Royal Infirmary, Edinburgh, had already been abandoned because of such difficulties. Gustavsson *et al* (1979) published an isotacophoretic method for the estimation of 5-fluorouracil, but this too is complex and time consuming. De Leenheer *et al* (1974) reported a simple column chromatographic method for the estimation of plasma 5-fluorouracil concentrations in which 5-fluorouracil is separated from plasma proteins on a 'Sephadex' G10 column and 5-fluorouracil is determined in the eluent simply by its UV absorbance at 266 nm. This method was investigated and proved to be very successful; recoveries of 5-fluorouracil from the column were good (96%) and 5-fluorouracil estimations were reproducible (C.V. 3.2%, n = 10). However, the

method had two characteristics which made it unsuitable for use in this study. The sensitivity was low such that the pharmacokinetic study would involve removal of 25% of the total blood volume of the rat. Also, each estimation took over 4 hours to complete.

Therefore a method was developed in which plasma samples were deproteinized by ultrafiltration through an 'Ultrafree' drug filter (Millipore (U.K.) Ltd., London) before measurement of absorbance at 267 nm. The filters, described below, are normally used to determine free, as opposed to bound, anticonvulsant drug concentrations in plasma. Control experiments and recovery experiments indicated the general suitability of the method. Furthermore, estimations could be made on as little as 0.3 ml of blood and in only 30 minutes. Therefore this method was adopted.

Pharmacokinetic studies

Plasma concentrations of 5-fluorouracil were determined during the first 4 hours after an intraperitoneal injection of either salt of 5-fluorouracil. Since the plasma concentrations of 5-fluorouracil after an intraperitoneal injection are determined both by the rate of absorption of the drug into the blood stream and by the rate of clearance of the drug from the blood, clearance was studied independently after an intra-arterial injection of each salt of 5-fluorouracil.

The results from this investigation have been accepted for publication (Plumb and Gardner, 1981).

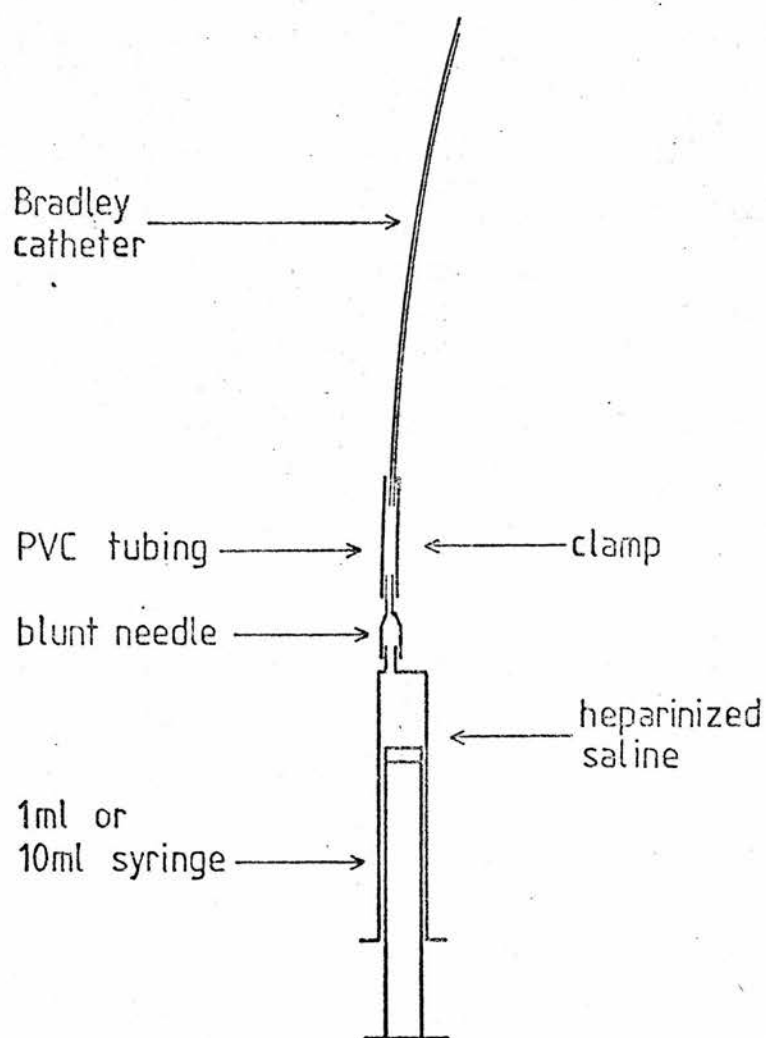


Fig. 9.1 The apparatus used for cannulation of the carotid artery.

A section of tubing (about 10 cm) cut from a Bradley miniature catheter is connected via a short section of flexible tubing and a blunt needle to a 10 ml syringe. The syringe and the dead space of the tubing is filled with about 5 ml of heparinized saline and when in position in the artery, the arterial pressure is opposed by a clamp placed on the section of flexible tubing.

METHODS

Carotid artery cannulation

Female rats, weighing 195 - 205 g,* were anaesthetised 1 hour before use by subcutaneous injection of urethane (1.2 ml of 0.8 mg/ml solution) while under temporary light ether anaesthesia. With the rat supine on a pad of cellulose wadding B.P.C. ('Cellozene' supplied by Robinsons of Chesterfield) and head nearest the operator, a small area (about 1 cm square) of the neck skin was raised with forceps and excised. The subcutaneous tissue was dissected apart with forceps to reveal the salivary glands and neck muscles. The left carotid artery was located by blunt dissection beneath the sternohyoideus and omohyoideus muscles and adjacent to the trachea. The artery was separated from the nerve by blunt dissection with two pairs of forceps. A loop of fine thread (Dewhurst 'Silko' 50) was passed under the carotid artery and the loop cut. Two loose ligatures were tied and one placed at each end of the exposed section of the artery. One was tightened at the distal end of the isolated section. The artery was clamped proximally, about 1 cm from the distal ligature, with a pair of fine artery forceps which had short sections of PVC tubing over the clamps to prevent damage to the artery. The cannula comprised of a short section (about 10 cm) of tubing cut from a Bradley miniature catheter (Portex) about 0.5 mm diameter (see Fig. 9.1). One end was bevelled at an angle of 45° and the other was connected via a short section (about 3 cm) of

* For these studies alone, special care was taken to use rats of particularly similar weights.

'Tygon' PVC pump tubing (Technicon) and a blunt needle to a 10 ml syringe. The syringe was filled with 5 ml of heparinized bicarbonate saline (0.1 g sodium heparin, Sigma Grade 1, in 20 ml of a solution containing 120 mmol/l NaCl and 25 mmol/l NaHCO_3). A small amount was expressed into a beaker of saline with the open end of the cannula below the surface of the liquid. Once the entire cannula was filled with saline, the tip of the cannula was removed slowly from the saline so that the meniscus was held at the tip of the cannula. This ensured that no air was injected into the artery upon cannulation. The isolated section of the artery was supported by a pair of open dental packing forceps and a tiny incision was made in the artery wall, close to the distal ligature, with a pair of fine sharp scissors held at an angle of 45° to the length of the artery. The cannula was passed into the artery with the aid of the bevelled tip and a tight ligature was tied around the artery and cannula. The clamp was then removed and the arterial pressure opposed by pressure on the syringe plunger, although no saline was injected at this stage. The artery forceps were then clamped round the short section of flexible tubing on the cannula to prevent blood flow.

The neck incision was then covered by a small piece of 'Cellosene' dipped in saline and the rat was kept warm with an overhead lamp. Arterial cannulation was accomplished in less than 5 minutes.

Blood sampling

Blood samples were taken from the carotid artery via the cannula. The 10 ml syringe was replaced by a 1 ml syringe in which the dead space had been filled with heparinized saline, the clamp

was removed and 0.3 ml of blood withdrawn. The blood sample was heparinized with the heparin already present in the cannula and in the dead space of the syringe (about 0.05 ml and it was the same for all samples). The clamp was then replaced, the syringe removed and the 10 ml syringe replaced with its eccentric luer fitting downwards. The clamp was again removed and the syringe plunger withdrawn gently to remove the inevitable air bubble from the cannula. About 0.3 ml of heparinized saline was injected via the cannula and the clamp was replaced. The whole procedure could be carried out in about 5 seconds.

A clamp on the cannula to allow blood sampling was chosen in preference to a tap. This method has the advantage of a very small dead space which was essential in view of the small size of the blood samples.

5-Fluorouracil administration

The Tris salt of 5-fluorouracil was diluted 1:1 with the Tris placebo just before use so that equal volumes of the Tris and sodium salts were administered. The effect of administration volume is not known but it may be important, especially since the replacement saline did not contain colloid.

(a) Intraperitoneal injection. Preliminary studies indicated a need to minimise variability in the site of injection and a need to avoid injection into the intestine. Therefore 5-fluorouracil was administered via a laparotomy. The position was standardised to the middle of the lower left abdomen. A small piece of skin was cut away and a 'Medicut' intravenous cannula inserted (Aloe Medical,

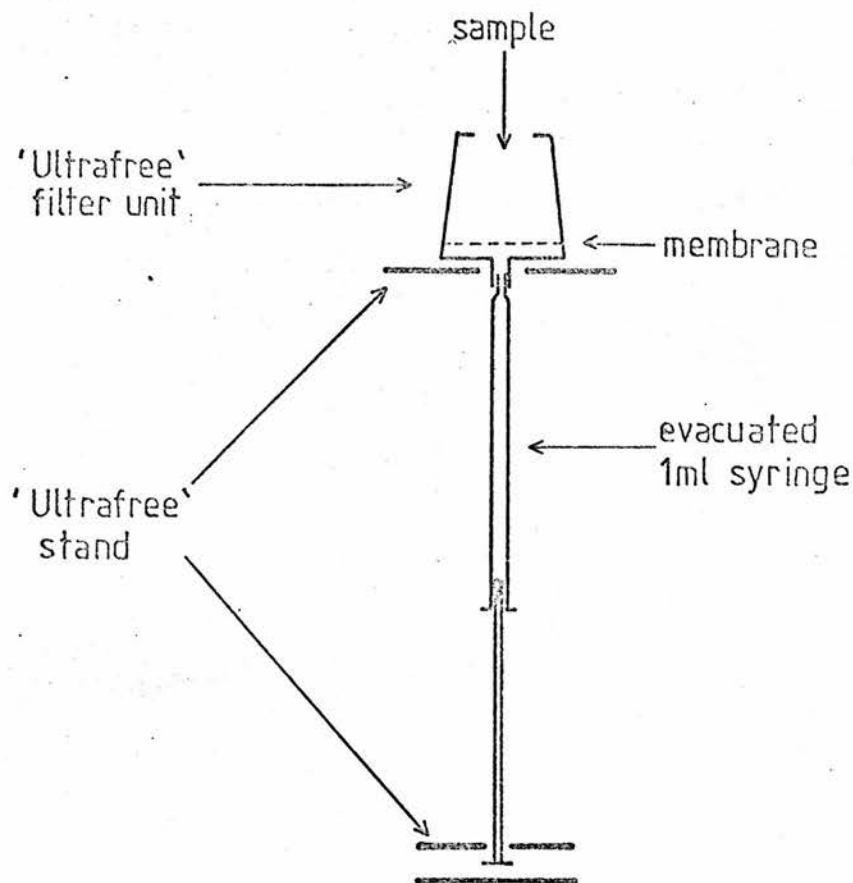


Fig. 9.2 Ultrafiltration of plasma samples through an 'Ultrafree' filter. A 1 ml syringe, with the plunger as far into the syringe as it will go, is attached to the luer fitting in the base of the filtration unit and the unit is placed in the 'Ultrafree' stand. The plasma sample is poured into the cup and allowed to diffuse into the membrane for about 2 minutes before the syringe plunger is withdrawn maximally and clamped into the lower part of the stand. The negative pressure produced in the syringe draws the filtrate through the membrane and into the syringe. Approximately 20 minutes is required for production of 1 ml of ultrafiltrate.

St. Louis, U.S.A.) angled towards the centre of the diaphragm. 5-fluorouracil (1.44 mmol/kg body weight) was injected via the cannula and the cannula withdrawn. The skin was closed by a single stitch.

(b) Intra-arterial injection. 5-fluorouracil (0.576 mmol/kg body weight, i.e. 0.6 ml/rat) was administered via the carotid artery cannula. The 5-fluorouracil injection was followed immediately by 0.2 ml of heparinized saline to wash out the cannula.

Injection via the sampling cannula is not ideal but was preferable to the alternative of a second arterial cannulation. It was justifiable in this instance because the carotid artery was cannulated close to the branch from the aorta. Thus all of the injected drug enters the aorta and should be rapidly dispersed throughout the blood volume.

Estimation of 5-fluorouracil

Blood samples were transferred to 4 ml capped plastic tubes and centrifuged at about 4000 g for 5 minutes at room temperature in an M.S.E. bench centrifuge. 5-fluorouracil was determined by the absorbance at 267 nm after ultrafiltration of the plasma, diluted at least ten-fold with pH 7.0 potassium phosphate buffer (291 ml 0.1 mol/l NaOH + 500 ml 0.1 mol/l KH_2PO_4 as used in the method of De Leenheer et al (1974) for the column chromatography of 5-fluorouracil) through an 'Ultrafree' filter (see Fig. 9.2 for details). Control plasma treated in this way showed no absorbance at 267 nm read against a blank of the potassium phosphate buffer. The buffer was used to standardise the pH at which absorbance was determined.

Absorbance was measured in a 'Unicam' SP500 spectrophotometer.

5-Fluorouracil calibration curve

A calibration curve was prepared for each salt of 5-fluorouracil in the range 2.5 - 12.5 ng/ml (0.019 - 0.096 $\mu\text{mol/l}$) in pH 7.0 buffer. The curve was linear in this range (Fig. 9.3) and there was no difference between the two salts. The concentrations of 5-fluorouracil in samples were determined from the first order regression line of the calibration curve.

Recovery of 5-fluorouracil from plasma

A calibration curve was prepared from a stock solution containing 250 ng 5-fluorouracil/ml plasma. The samples were ultra-filtered and the absorbance, measured at 267 nm, were compared with those from plasma free samples. The results for the sodium salt are shown in Fig. 9.4. The total recovery was $99.5\% \pm 3.9\%$ ($n = 5$). A similar value was obtained for the Tris salt ($99.1\% \pm 4.1\%$ $n = 5$).

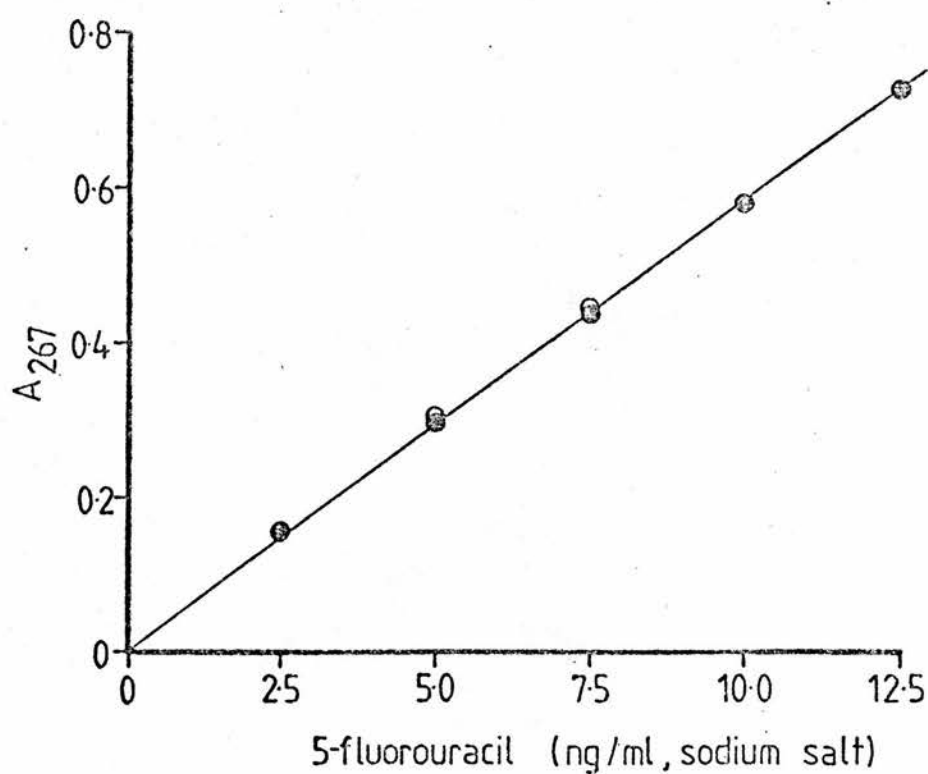


Fig. 9.3 Calibration curve for assay of 5-fluorouracil in the ultrafiltrate of plasma. Absorbance was measured at 267 nm with a 1 cm light path. The first order regression line has been drawn through the points ($n = 2$ for each concentration).

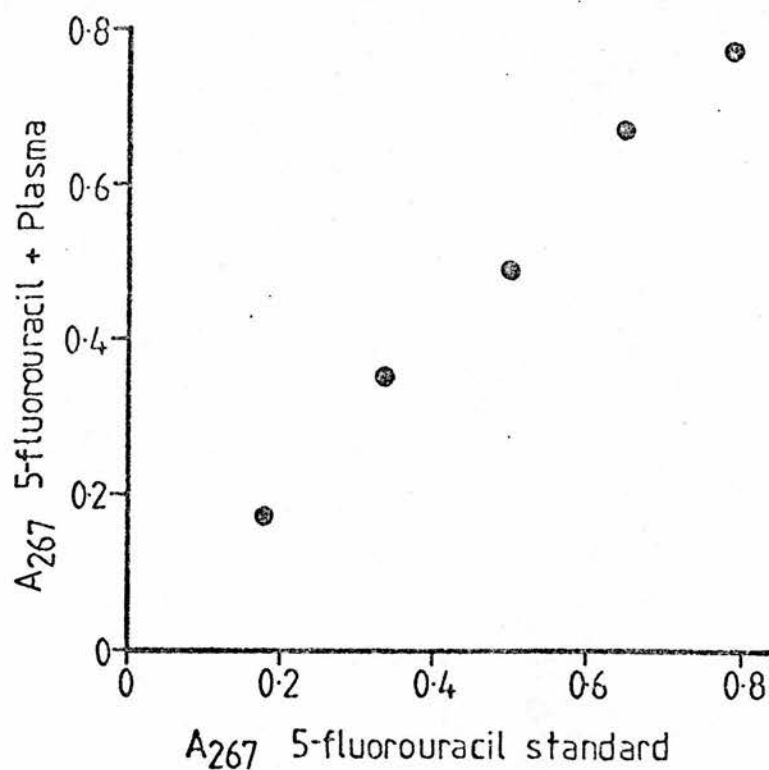


Fig. 9.4 Recovery of 5-fluorouracil from plasma. Calibration curves were prepared from a stock solution of 5-fluorouracil and from a stock solution containing 250 ng 5-fluorouracil/ml plasma. The absorbance of the latter samples after ultrafiltration (ordinate) are plotted against the absorbance of the former samples (abscissa).

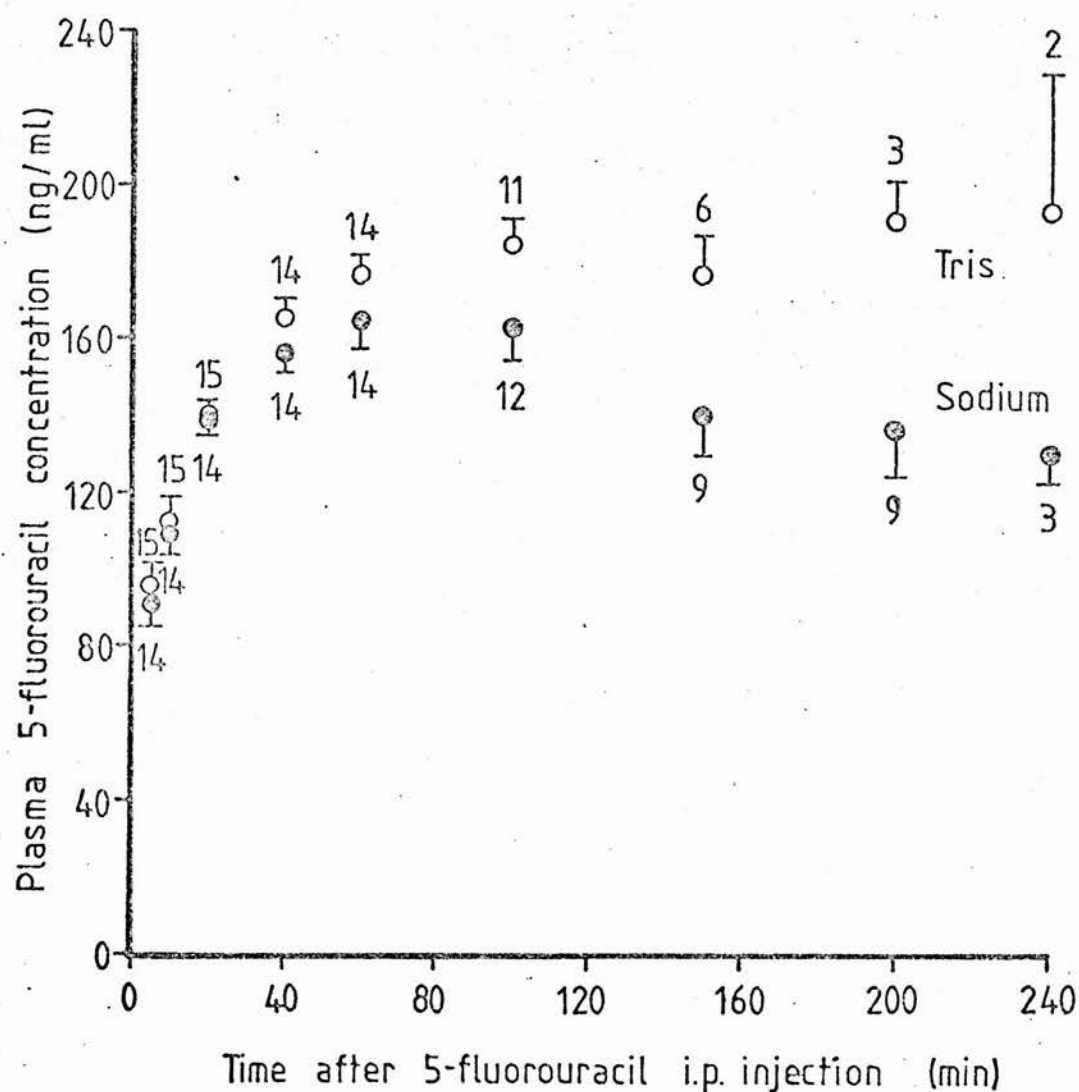


Fig. 9.5 Plasma levels of 5-fluorouracil after intraperitoneal injection of either the sodium salt (●) or the Tris salt (○) in anaesthetised female rats. Values are the mean \pm S.E.M. and the number of observations is shown adjacent to each point.

RESULTS

Intraperitoneal injection

Fig. 9.5 shows the plasma levels of 5-fluorouracil over the first 240 minutes following a single intraperitoneal injection of either the Tris or the sodium salt of 5-fluorouracil. Plasma levels of 5-fluorouracil were consistently higher after injection of the Tris salt than after injection of the sodium salt, although this difference was only just significant, by a 't test', at the individual points corresponding to 100, 150 and 200 minutes after injection ($P < 0.05$). However, the fact that at each of the sampling times the plasma 5-fluorouracil concentration was greater after administration of the Tris salt than after administration of the sodium salt indicates that there is a real difference between the two curves. This has been tested by a statistical test which combines the probabilities of all observations in an overall test of the significance of the difference between the two curves (Fisher, 1967) and, indeed, the difference is significant ($P < 0.02$).

The number of observations decreased with time, especially after administration of the Tris salt of 5-fluorouracil, due to deaths during the experiment. It is possible that mortality might have been systematically associated with higher plasma 5-fluorouracil levels. Therefore the results shown in Fig. 9.5 were replotted as a series of curves, each representing the mean plasma levels observed in a group of rats, each of which survived for longer than a specified period (Fig. 9.6). Removal of observations in this manner had no significant effect on the mean plasma levels of 5-fluorouracil, although most were slightly increased.

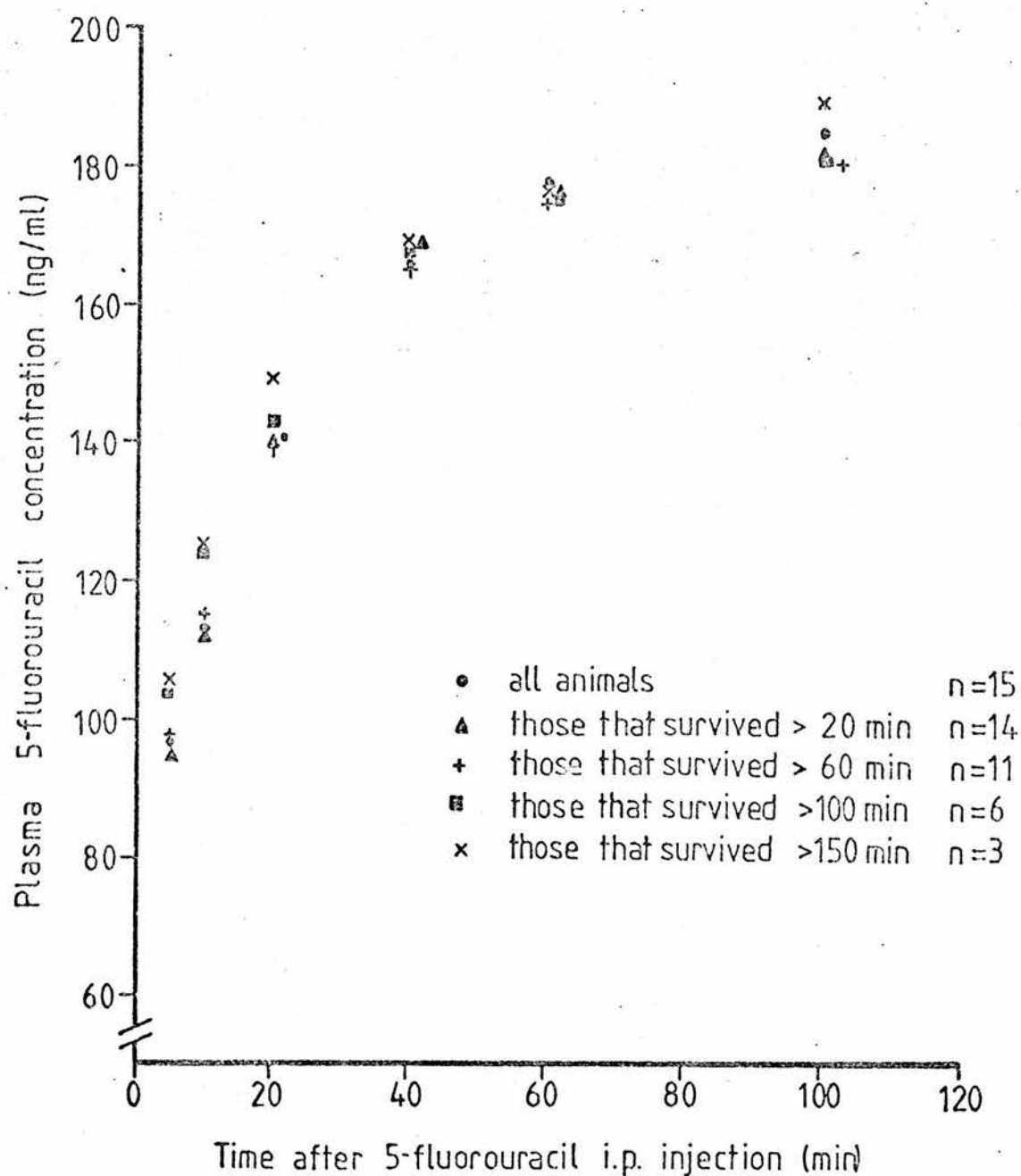


Fig. 9.6 Plasma levels of 5-fluorouracil after intraperitoneal injection of the Tris salt of 5-fluorouracil. The results shown in Fig. 9.5 have been re-plotted to exclude animals that died at various times during the period of observation.

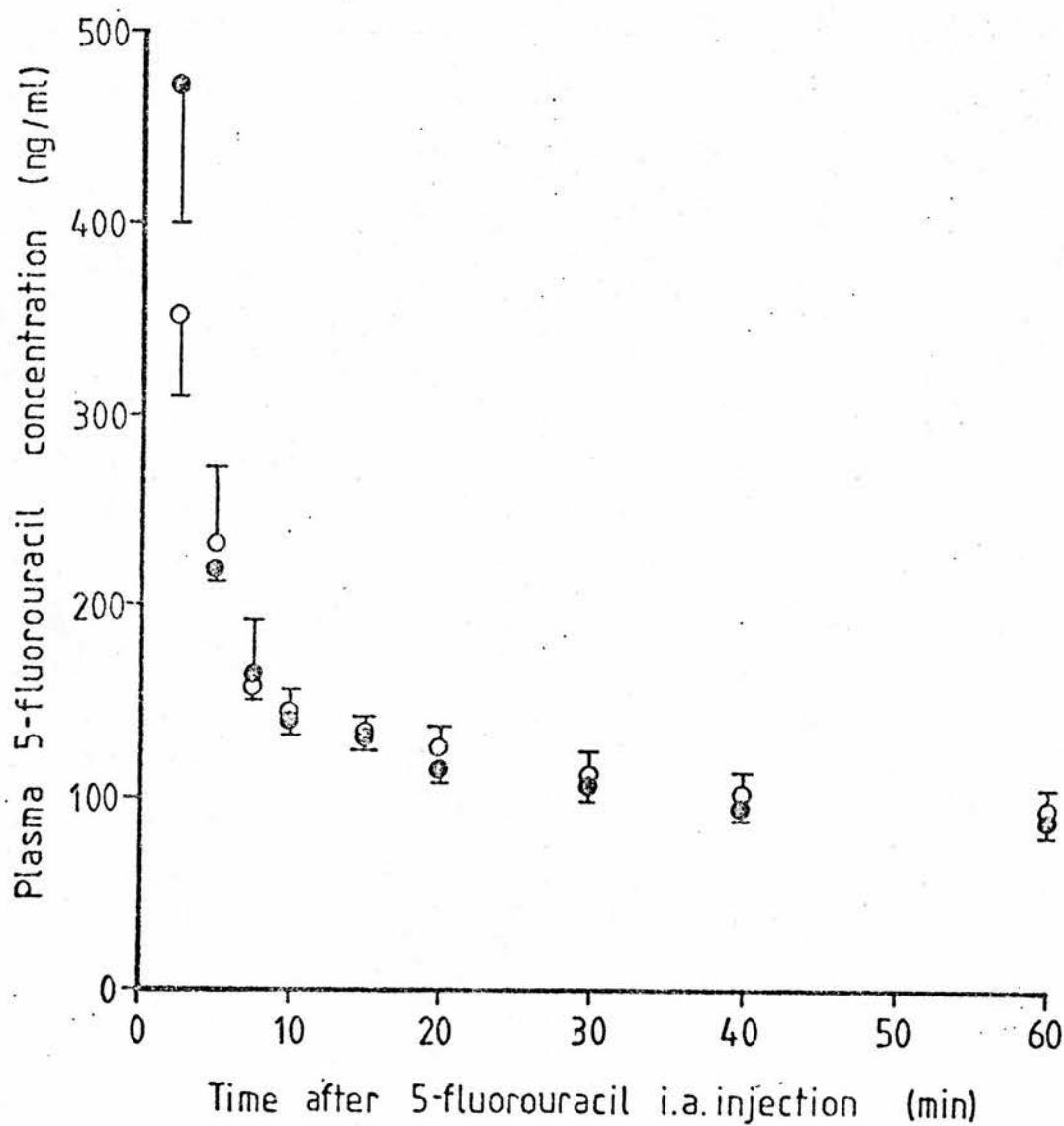


Fig. 9.7 Plasma levels of 5-fluorouracil after intra-arterial injection of either the sodium salt (●) or the Tris salt (○) in anaesthetised female rats. Values are the mean \pm S.E.M. and each point is the mean of 3 or 4 observations after sodium or tris. salt injection respectively.

Intra-arterial injection

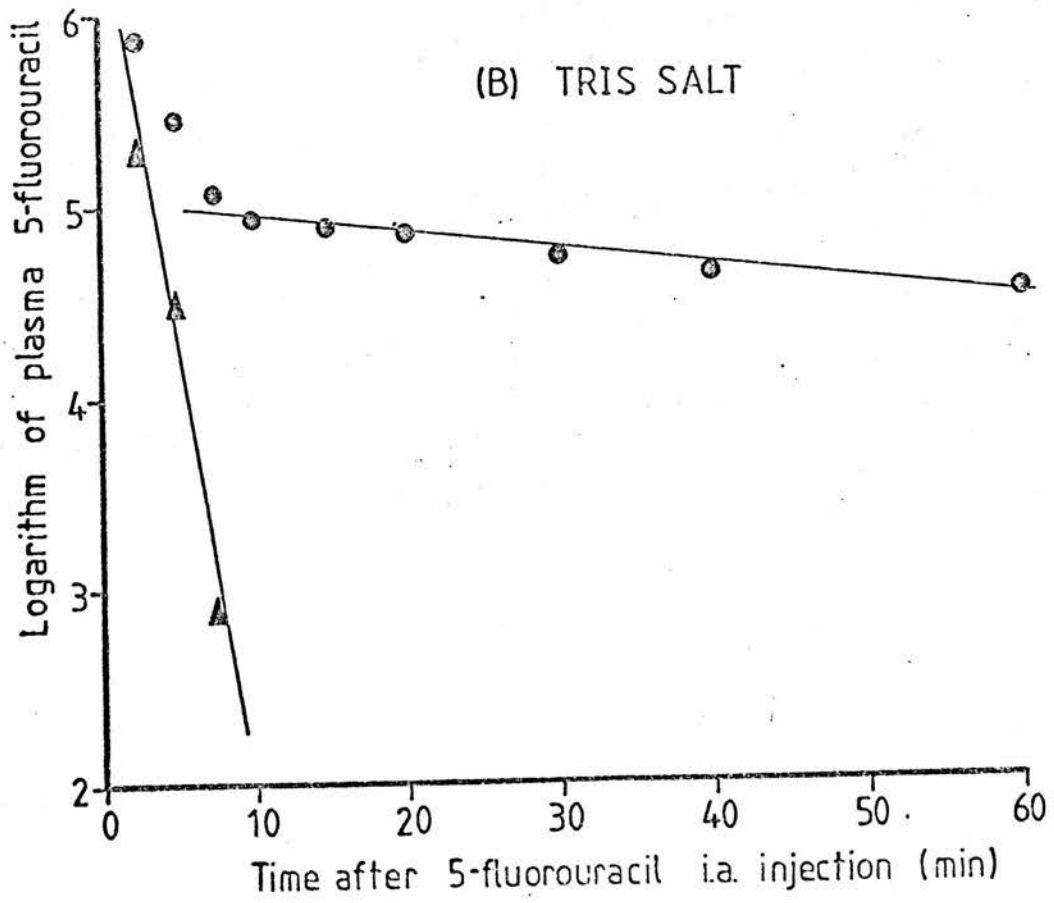
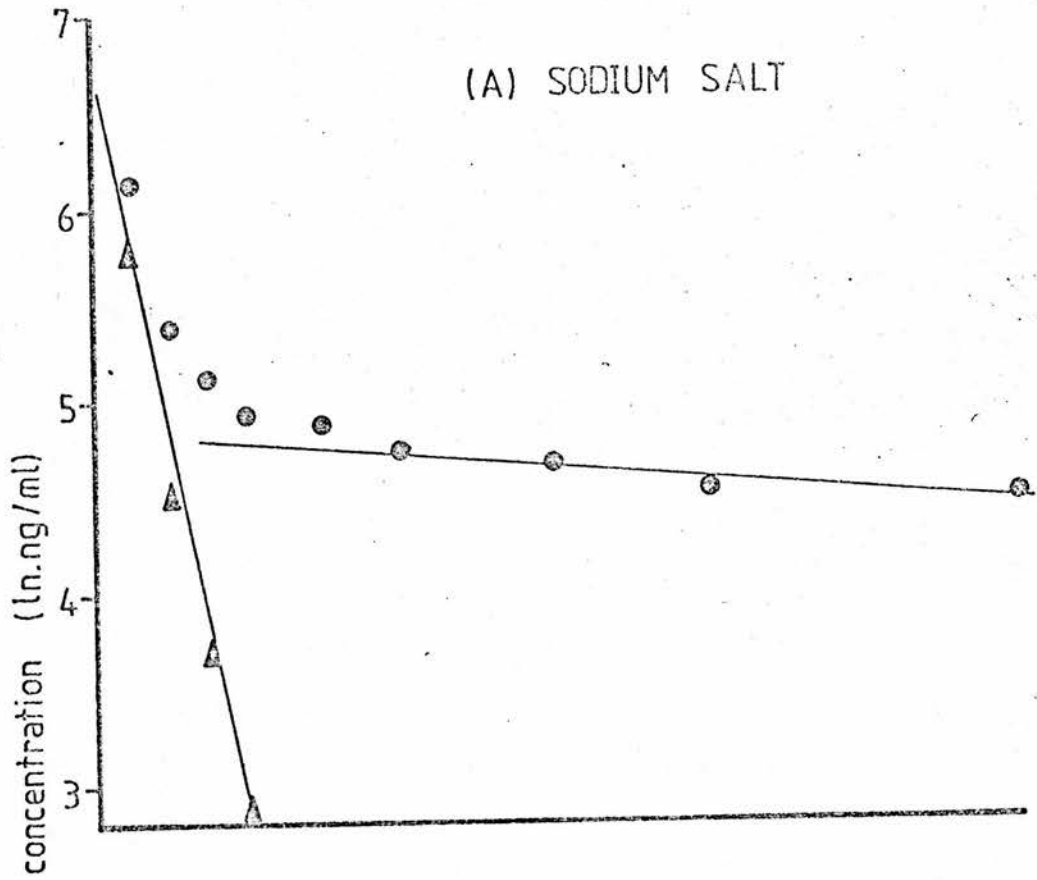
Fig. 9.7 shows the plasma levels of 5-fluorouracil during the first 60 minutes after intra-arterial injection of the Tris or the sodium salt of 5-fluorouracil. Plasma levels of 5-fluorouracil were very similar at all times after injection of the two salts of 5-fluorouracil. There was a very rapid clearance of 5-fluorouracil during the first 10 minutes and the estimations of plasma levels of 5-fluorouracil during this period were associated with high variability. Plasma levels of 5-fluorouracil decreased very slowly over the next 50 minutes.

The curves shown in Fig. 9.7 can be described by the sum of two exponentials. Fig. 9.8 shows the separation of these two exponentials for the Tris and sodium salts. The natural logarithm of the plasma 5-fluorouracil concentration is plotted against the time after injection of 5-fluorouracil (●). The parameters of the exponential term with a slow rate constant were obtained from the regression line through the last four points for each curve. By extrapolation of the exponential back to time 0, the expected plasma concentrations of 5-fluorouracil for the first three or four observations were calculated. These values were subtracted from the observed plasma concentrations and the natural logarithm of the differences plotted in Fig. 9.8 (▲). The regression line through these points gave an estimate of the parameters of the second exponential term. These were not very reliable due to the small number of points.

This method produced the following two equations for the curves:

Tris salt; plasma 5-fluorouracil concentration = $131.3 e^{-0.007t} + 787.3 e^{-0.39t}$

Fig. 9.8 Separation of the two exponential functions which describe the results shown in Fig. 9.7. The natural log of the plasma 5-fluorouracil concentration is plotted against the time after injection of 5-fluorouracil (\circ). The parameters of the exponential term with a slow rate constant were obtained from the regression line through the last four points for each curve. By extrapolation back to time 0, the expected plasma concentrations of 5-fluorouracil for the first 3 (Tris salt) or 4 (sodium salt) observations were calculated. These values were subtracted from the observed plasma concentrations and the natural log of the difference is also plotted (Δ). The regression line through these points gives an estimate of the parameters of the second exponential term.



sodium salt; plasma 5-fluorouracil = $148.6 e^{-0.008t} + 790.7 e^{-0.48t}$
concentration

and the two half lives for the Tris salt were 1.8 and 99 minutes
and for the sodium salt were 1.4 and 86 minutes.

Curve fitting

An attempt was made to analyse the results obtained after intra-arterial injection of 5-fluorouracil by curve fitting with the aid of a digital computer program for fitting the sums of decaying exponentials by non-linear regression analysis. The program, described by Atkins (1971), requires initial estimates of the parameters and uses an iterative procedure to adjust the parameters until the sum of squares of residuals has converged to a minimum. The program was run on the ICL 2980 computer at the Edinburgh Regional Computing Centre. The data were entered unweighted and the following best-fit equations were obtained:

Tris salt;

$$\begin{aligned} \text{plasma 5-fluorouracil} &= (178.6 \pm 19.5)e^{-(0.013 \pm 0.005)t} \\ \text{concentration} &+ (1579.9 \pm 23.5)e^{-(0.835 \pm 0.045)t} \end{aligned}$$

sodium salt;

$$\begin{aligned} \text{plasma 5-fluorouracil} &= (155.6 \pm 8.4)e^{-(0.011 \pm 0.002)t} \\ \text{concentration} &+ (1582.2 \pm 8.4)e^{-(0.628 \pm 0.011)t} \end{aligned}$$

and the two half lives for the Tris salt were 0.83 and 53.7 minutes
and for the sodium salt were 1.1 and 64.8 minutes.

These parameters compare moderately well with those obtained by the graphical method and both methods demonstrate the similarity

between the plasma pharmacokinetics of the Tris and sodium salts of 5-fluorouracil after intra-arterial injection.

An attempt to analyse the results obtained after an intra-peritoneal injection by curve fitting was unsuccessful, thus suggesting that neither a two or three compartment exponential model was appropriate.

DISCUSSION

That the Tris and sodium salts of 5-fluorouracil have such remarkably different systemic toxicities in the rat was both interesting and puzzling. This observation suggested either that the cytotoxicity of the Tris salt was greater than that of the sodium salt or that the two salts of 5-fluorouracil were associated with different pharmacokinetics.

Therefore, the plasma pharmacokinetics of the two salts have been studied and, indeed, there is a difference after intraperitoneal injection. Higher plasma levels were achieved and sustained after injection of the Tris salt than after injection of the sodium salt of 5-fluorouracil (Fig. 9.5) and this difference was significant ($P < 0.02$).

In contrast, when 5-fluorouracil was administered by the intra-arterial route, the pharmacokinetics of the two salts were very similar (Fig. 9.7). Clearance of 5-fluorouracil from the blood can be described by a two-exponential equation combining a very rapid clearance with a much slower process. Similarly, Kirkwood *et al* (1980) and Finch *et al* (1979) have reported that plasma clearance of 5-fluorouracil after intravenous injection in the human could be described as a biexponential decay function. As in the rat, the clearance of plasma 5-fluorouracil in the human involved a rapid ($t_{1/2} = 7.8$ minutes) and a slow ($t_{1/2} = 36.8$ minutes) process. Rough estimates were obtained for the two half lives of these processes in the rat and these were of the same order as those in the human (about 1.6 and 93 minutes or 0.97 and 59.3 minutes depending on the method used). However, since Finch *et al* (1979) demonstrated that

that the plasma kinetics of 5-fluorouracil are dose dependent direct comparisons must not be made.

The dose dependence of the pharmacokinetics cannot explain the difference between those of the Tris and sodium salts of 5-fluorouracil, since identical doses were used and since the difference was apparent only following intraperitoneal injection of the drug. That the difference was seen following intraperitoneal injection and not after intra-arterial injection also confirms that the difference was a real one, and was not due to some characteristic of the technique used to study the pharmacokinetics.

Since the clearance of 5-fluorouracil from plasma after intra-arterial injection was independent of the salt of 5-fluorouracil administered, the difference observed after intraperitoneal injection was probably due to a more rapid uptake of the Tris salt from the peritoneal cavity rather than due to a slower clearance of the Tris salt from the blood. Drug absorption from the peritoneal cavity may occur by diffusion of the non-ionised form of the drug across the lipid membrane of cells. Therefore, it may be relevant that Tris base is a weaker base ($pK_a = 8.1$ at 25°C - Dawson *et al*, 1969) than sodium hydroxide. Hence the Tris salt of 5-fluorouracil is likely to be less dissociated, and thus may permeate some membranes more rapidly according to current theories of non-ionic diffusion (Rowland, 1972). In this respect it may be relevant that the Tris and sodium salts were administered at a slightly different pH; that of the Tris salt was 8.8 and that of the sodium salt was 9.1 (Chapter 8, Table 8.3), and this might have influenced the dissociation of the two salts. However, the pH of the peritoneal

fluid is maintained by the buffering capacity of the blood and it is probable that the pH of the injected fluid would be rapidly equilibrated with that in the peritoneum. Nevertheless, it would have been preferable to have administered the two salts at exactly the same pH as well in order to eliminate this possibility.

These observations suggest two possible explanations for the differential toxicities of the two salts of 5-fluorouracil:

- (1) The higher plasma levels of 5-fluorouracil after intraperitoneal administration of the Tris salt might explain directly the differential toxicities of the Tris and sodium salts of 5-fluorouracil; or
- (2) There is a fundamental difference between the properties of the Tris and sodium salts of 5-fluorouracil, and that it is a property of the Tris salt per se that is responsible for the severe systemic toxicity and mortality associated with its use. .

The first alternative would imply that this differential toxicity would not be seen with intra-arterial or intra-venous dosage - the routes generally used in clinical practice. In this case these observations, although interesting, would not be relevant to clinical chemotherapy. On the other hand, Speyer et al (1980) (see also Dedrick et al, 1978) have advocated the use of 5-fluorouracil in peritoneal dialysis in the chemotherapy of various metastatic tumours; the observations discussed above would be relevant to this method of administration of 5-fluorouracil in

humans. However, mortality and the systemic toxicity of the Tris salt were equally severe when the dose was reduced from 1.44 mmol/kg body weight to 1.08 mmol/kg (Chapter 8, Fig. 8.8). Since Speyer et al (1980) showed that the rate of clearance of 5-fluorouracil from the peritoneal cavity is directly dependent upon the concentration of 5-fluorouracil in the peritoneal fluid, this alternative is considered to be unlikely. Observations on the systemic toxicities of the two salt forms following intra-arterial or intra-venous administration are required before a definite conclusion can be drawn.

It is therefore possible that there is a fundamental difference between the properties of the Tris and sodium salts of 5-fluorouracil. The results suggest faster uptake of the Tris salt across the peritoneum; the same may be true of some other membranes, although it is not true for the overall tissue uptake, since differences in plasma clearance were not seen after intra-arterial administration. However, this might explain the pancreatic damage observed specifically after administration of the Tris salt of the drug (Chapter 8).

It is important that the cause of the differences between the Tris and sodium salts of 5-fluorouracil is resolved, especially since the clinical use of 5-fluorouracil is severely limited by the toxic side effects of the drug. Although the Tris salt of 5-fluorouracil is no longer in clinical use, these observations on the differential toxicities of the two salts may be of relevance to the clinical use of 5-fluorouracil, especially in the U.K. where the sodium salt has only been in use since 1978. Much of the

knowledge of the side effects of 5-fluorouracil has, therefore, been gained from experience with the Tris salt of 5-fluorouracil, and this may not apply to the sodium salt.

CHAPTER 10

Can 'elemental' diets reduce the intestinal toxicity of

5-fluorouracil?

Introduction

So-called 'elemental' diets originate from efforts to produce a chemically defined diet, composed of relatively simple nutrients requiring minimal digestion, that would nevertheless enable humans to grow, reproduce and maintain a satisfactory nutritional status. This was first achieved by Rose and his associates in the late 1940's with a diet composed of corn starch, sucrose, butterfat, corn oil, inorganic salts, vitamins and hydrolysed casein (Rose, 1949; Rose and Wixom, 1955). These diets are not 'elemental' in the strict sense; some people prefer to use the term 'chemically defined diets' but this, too, is not correct for diets containing partial, e.g. enzymic, hydrolysates of protein.

The American aerospace programme initiated great interest in the development of 'elemental' diets as a possible source of nutrition for astronauts; hence the references to 'elemental' diets as 'space-diets'. These diets have a high nutritional value and therefore only small amounts are required daily, an important consideration where storage space and weight are limited. Also, the low residue of the diets considerably reduces the faecal output and thus reduces the problems of waste disposal.

The composition of 'elemental' diets is very flexible and in theory can be adjusted to suit any specific nutritional requirements. Several 'elemental' diets are commercially available in the U.K.;

two are used on a large scale and, therefore, have been used in the present work. These are 'Flexical' (Mead-Johnson, Bristol Laboratories, Langley, Slough, U.K.) and 'Vivonex-standard' or 'Vivonex-HN' (Eaton Laboratories, Woking, Surrey, U.K.) and they differ markedly in their composition - see below. Two others, 'Pregestimil' and 'Nutramigen' (both Mead-Johnson) are formulated for use in infants.

Since it is now known that both amino acids and small peptides are absorbed from the intestinal lumen by independent mechanisms (Craft *et al*, 1968; Asatoor *et al*, 1970; Matthews, 1972; Milne, 1972) and that the absorption of some amino acids may be more rapid when they are presented as small peptides rather than as free amino acids (Matthews, 1975; Silk, 1977) it is not necessarily an advantage to include only free amino acids in 'elemental' diets. In the human, similar proportions of each of the constituent amino acids are absorbed from an enzymic hydrolysate of casein, but the proportions differ when the hydrolysate is replaced by an amino acid mixture simulating casein (Silk, 1977). Also there is evidence which suggests that the growth rate of animals is not as good on mixtures of free L-amino acids as on protein with the same amino acid composition (Fisher, 1954, p. 104). Furthermore, enzymic hydrolysates exert a lower osmotic pressure than equivalent solution of free amino acids and thus are less liable to cause the 'dumping syndrome'. It is possible, therefore, that 'elemental' diets should contain an enzymic hydrolysate as the nitrogen source rather than only free amino acids.

The compositions of the two commercially available 'elemental'

diets used in this study are very different. A comparison of the distribution of calories between carbohydrate, fat and amino-nitrogen of 'Vivonex-standard', Vivonex-HN and 'Flexical' is shown in Table 10.1. Free amino acids are the nitrogen source in the two

Table 10.1 A comparison of the compositions of 'Vivonex',
'Vivonex-HN' and 'Flexical'

Component	% of total calories		
	'Vivonex'	'Vivonex-HN'	'Flexical'
Carbohydrate	90.2	81.0	61.0
Free amino acids	8.5	18.3	6.3
Small peptides	0	0	2.7
Fat	1.3	0.8	30.0

'Vivonex' formulations, representing either 8.5% of the total calories in 'Vivonex-standard' or 18.3% in 'Vivonex-HN'. In 'Flexical' 9.0% of the calories are supplied by an enzymic hydrolysate of casein in which 70% of the nitrogen is present as free amino acids and 30% as small peptides. The size of the peptides is not specified but they are dialysable and are probably between 2 and 4 amino acids. All but 1% of the rest of the calories in 'Vivonex' are supplied by carbohydrate in the form of glucose and small oligosaccharides. The remaining 1% of the calories are present as highly purified safflower oil (80% as triglyceride of linoleic acid). 'Flexical', in contrast, contains much less carbohydrate, 61% of the total calories, in the form of sucrose and small oligosaccharides and contains a large amount of fat, 30% of the total

calories. Of the fats present, soy oil, soy lecithin and medium chain triglycerides, the latter do not require micelle formation and therefore, in principle, should be easily absorbed (Kaunitz, 1971). To-date, however, there have been no direct comparative studies of the two diets, and so there is no evidence to suggest that either diet has a clinical advantage over the other.

The diets are supplied as dry powders and are consumed as a solution or suspension in water, as a jelly or as a solution in water frozen to form a solid 'ice'. A variety of flavours can be added to the diets but, as stated by Pestana (1977, p. 141), these "modify only slightly the basic taste of rotten fish" due to the presence of some free amino acids and peptides. Because of this many patients find the diets intolerably unpalatable and this apparently was the reason why they were abandoned as the source of nutrition for astronauts (Koretz and Meyer, 1980). In order to overcome unpalatability of the diets they may be fed by a nasogastric tube, but this too is unpleasant and can be associated with problems such as aspiration. At the standard dilution the diets have a very high osmolarity (840 mOsm/l for 'Vivonex-HN') due to the high content of low molecular weight carbohydrate and this can result in several undesirable side effects such as nausea, diarrhoea, delayed gastric emptying and the 'dumping syndrome', especially in susceptible individuals. The osmolarity of the diets can be reduced by replacement of monosaccharides by disaccharides or small oligosaccharides, or by further dilution of the diet. The latter method has the disadvantage of increasing the volume of diet to be consumed. One of the main complications of the use of 'elemental' diets is reported to be involved with the osmolar load

and the rate of administration of the diet (Young et al, 1975).

Clinical trials with 'Flexical' and with diets similar to 'Vivonex' suggest that humans can be maintained in good health for periods of at least 22 weeks with no apparent adverse effects (Perrault et al, 1973; Winitz et al, 1970). However, there have been reports of pancreatic damage and haemolytic anaemia when 'elemental' diets containing free amino acids were fed to rats for prolonged periods (Geever et al, 1970; Levenson et al, 1971). Since damage was not observed in rats fed on 'elemental' diets containing enzymic hydrolysates of protein, this suggests that there is an advantage from inclusion of peptides in 'elemental' diets, although it is hard to provide a rational explanation.

Many patients with a number of acute and chronic diseases and many undergoing surgery develop a negative nitrogen balance due to a high rate of protein catabolism (Cuthbertson, 1936; Moore, 1959). Intravenous administration of mixtures of hydrolysed protein and glucose has been used with considerable success during the surgical treatment of many diseases (Dudrick et al, 1968; Dudrick and Rhoads, 1971). However, intravenous administration of nutrients requires a central catheter, is costly and is associated with the risks of sepsis due to the intravenous catheter and with metabolic acidosis. Therefore successful enteral nutrition in such patients would have considerable advantages. Recently 'elemental' diets have been used in a number of clinical conditions. The diets can be fed orally and their 'elemental' nature should enable their use when the digestive process is severely impaired. They are absorbed almost entirely by the upper small intestine such that only the endogenous

residue (normally minimal) enters the large intestine.

Nutritional support from 'elemental' diets has been reported to benefit the recovery of patients with various gastrointestinal diseases such as acute inflammatory bowel disease and other diarrhoeal conditions and gastrointestinal fistulae (Bode and Hendren, 1970; Bury et al, 1969; Goode et al, 1976; Grundy, 1971; Pincus et al, 1971; Rocchio et al, 1974; Stephens and Randall, 1969; Voitk et al, 1973a; Wolfe et al, 1972). 'Elemental' diets have also been reported to provide adequate nutrition to patients who have undergone extensive gut resection where there is insufficient gut remaining to cope with the digestion and absorption of conventional foods (Thompson et al, 1969; Voitk et al, 1973b; Weinberger and Rowe, 1973). Furthermore, nutrition via the gastrointestinal tract may well be preferable to parenteral nutrition in these patients since the presence of food in the gut is said to be a necessary stimulus for mucosal growth following resection (Feldman et al, 1974). Since 'elemental' diets do not require the action of pancreatic proteases these diets have been used for the nutritional support of patients with pancreatic insufficiency or pancreatitis where the production of pancreatic proteases is reduced (Pincus et al, 1971; Stephens and Randall, 1969; Voitk, 1973c). Also, it has been suggested that the intra-jejunal use of 'elemental' diets avoids stimulation of pancreatic secretion and that this is favourable in the treatment of pancreatitis (Ragins et al, 1973).

In general, few of the uses discussed above have been assessed in adequate trials including appropriate controls and therefore

little can be concluded as to the real benefits, if any, of the use of 'elemental' diets in these patients. However, controlled studies have been carried out to determine whether the low residue nature of the diets is useful in colonic preparation for colonoscopy (Teague *et al*, 1973) or as a general bowel preparation (Cooney *et al*, 1974; Gurry and Ellis-Pegler, 1976; Johnson, 1974; Matheson *et al*, 1978; Tomlinson *et al*, 1976). All of these studies concluded that there was no advantage in the use of 'elemental' diets instead of the conventional method for bowel preparation.

In 1967 Bounous and his associates suggested a novel application of 'elemental' diets in the prophylaxis of experimental haemorrhagic shock and intestinal ischaemia (Bounous *et al*, 1967). The implications of this application (see below) are important and have attracted a great deal of attention. Bounous *et al* (1967) suggest that during the digestion and absorption of normal food the intestine is highly susceptible to ischaemia, and that an 'elemental' diet reduces this susceptibility.

Subsequently, Bounous *et al* (1971a) went on to claim that the intestinal damage observed on the first few days after administration of the antimetabolite 5-fluorouracil in rats was markedly reduced when the rats were fed on an 'elemental' diet. Three days after 5-fluorouracil administration the intestines from control rats, fed on a standard diet, showed total atony throughout the entire length, oedema, congestion and extensive necrosis. In contrast, those from rats fed on the 'elemental' diet showed normal tonicity in most cases with no oedema and the villi were preserved. The weight loss normally observed after 5-fluorouracil administration was not

observed in rats fed on an 'elemental' diet. Bounous et al (1971a) suggested that the diet is of benefit to the intestine during recovery from injury rather than in reducing the severity of the injury. This seemed to be plausible since intestinal cells are reported to incorporate into protein amino acids absorbed from the lumen to a greater extent than amino acids supplied systemically (Hirschfield and Kern, 1969). Since Bounous et al (1971a) reported a decrease in peptide hydrolase activities in the intestinal mucosa on the first day after 5-fluorouracil administration they suggested that the supply of amino acids to the mucosa from an unhydrolysed diet would be severely limited. On the other hand, an 'elemental' diet supplies amino acids directly to the mucosal cells without the need for digestion.

The intestinal damage resulting from irradiation is similar to that after 5-fluorouracil administration (Robinson, 1972). Hugon and Bounous (1972) claimed that the same 'elemental' diet provides a beneficial effect to the animal during irradiation. They showed that the 30 day survival of mice, following whole body irradiation, was increased when mice were fed on an 'elemental' diet; at high doses of irradiation (10 Gy) this benefit was not observed. They also claimed that at lower doses of irradiation (7 Gy) the 'elemental' diet stimulated a more rapid recovery of the intestinal mucosa. However, histological evidence indicated that the 'elemental' diet did not protect crypt cells from irradiation damage but may have protected the villus cells.

The 'elemental' diet 'Flexical' was developed from Diet 3200-AS used by Bounous et al (1971b) and was used in clinical trials

(Bounous et al, 1971b) before being marketed. A number of patients with advanced metastatic carcinomas of the gastrointestinal tract, lung or breast were fed either on normal hospital food or on 'Flexical' for 4 days before and during 5-fluorouracil chemotherapy. Patients fed on the 'elemental' diet did not suffer any weight loss nor the rectal lesions seen in patients given normal hospital food.

5-Fluorouracil administration in rats is followed by severe impairment of intestinal absorption of both glucose and water and of cytoplasmic peptide hydrolase activities (Gardner et al, 1978; Levin, 1968). When the normal diet was replaced by one of a number of 'elemental' diets, both commercial and home-made, the intestinal toxicity of 5-fluorouracil was of similar severity to that observed in the control rats, fed on a standard diet, from which Gardner and Heading (1979) concluded that a direct effect on intestinal absorption could not account for the beneficial effects of 'elemental' diets claimed by Bounous et al (1971a). However, Bounous and Maestracci (1976) noted that it is the replacement of intact protein by a protein hydrolysate in 'elemental' diets that is responsible for protecting the intestine from the toxic effects of 5-fluorouracil. If the protein hydrolysate content of the diet is reduced by 30% and the difference made up with intact protein, the beneficial effects of the diet are lost. The diets used by Gardner and Heading (1979) contained either free amino acids only ('Vivonex') or intact protein (casein or lactalbumin) or an enzymic hydrolysate of casein or lactalbumin. All the diets, except for 'Vivonex', contained a significant quantity of intact protein (about 6% by weight of the diet) present in the flour used in the diets. In contrast, the nitrogen source in the 'elemental' diets used by

Bounous et al (1971a and b) was both free amino acids and small peptides instead of protein (Diet B and 'Flexical'). Thus none of the diets used by Gardner and Heading (1979) are exactly comparable, in terms of the nitrogen source, to those used by Bounous et al (1971a and b) and this might explain the lack of effect observed by Gardner and Heading (1979).

Very little is known about the physiological effects of 'elemental' diets on the gastrointestinal system. Bounous et al (1971a) reported that mucosal peptide hydrolase activities were decreased in rats fed on an 'elemental' diet. However, Gardner and Heading (1979) showed that the effects of 'elemental' diets on intestinal peptide hydrolase activities depends also upon the actual nitrogen source in the diet and upon the particular enzymes whose activities are measured. Thus it is possible that the beneficial effects observed by Bounous et al (1971a and b) were due to some aspect of the diet other than its 'elemental' nature.

The claims made by Bounous and associates are of enormous significance: if the adverse effects associated with chemotherapy and irradiation can be reduced by simple dietary modification, this would be highly valuable in clinical practice. Apart from a single investigation of the therapeutic effect of an 'elemental' diet on proline absorption across the irradiated rat small intestine by Mohiuddin and Kramer (1978) (discussed on p.263) the work of Bounous and associates is the only evidence in favour of dietary modification to reduce 5-fluorouracil toxicity. Gardner and Heading's findings did not support Bounous's claim. Furthermore, Stanford et al (1977) concluded that the protein hydrolysate content of 'elemental' diets

was responsible for the increased toxicity of 5-fluorouracil that they observed in rats fed on an 'elemental' diet.

Therefore this chapter describes a study of the physiological effects of 'elemental' diets on the small intestine and of the effects of 'elemental' diets on the intestinal toxicity of 5-fluorouracil. The food intakes and growth rates of rats fed on three 'elemental' diets have been compared with those of rats fed on a standard diet as an indication of the suitability of the diets for the nutrition of rats. One of the diets used, Diet B, has a composition as close as could be achieved to that used by Bounous et al (1971a). Intestinal absorption, cytoplasmic peptide hydrolase activities and mucosal DNA contents have been estimated in rats fed on these 'elemental' diets and in rats fed on an 'elemental' diet before and after 5-fluorouracil administration. Intestinal water absorption rates have been used as the measurement of intestinal function, since water absorption correlates with glucose absorption (Gardner et al, 1978; Gardner and Heading, 1979) and thus it is the simplest index of absorptive activity. The diets have been fed for various times prior to 5-fluorouracil administration since there is no information available on the optimum duration of dietary treatment. Also, both male and female rats have been used, since Pageau et al (1977) reported that the effects of 'elemental' diets on the immune system differ between male and female rats. While Bounous et al (1971a) studied male rats, Gardner and Heading studied female rats, and this difference could be relevant in explaining the conflicting results reported.

METHODS

'Elemental' diets

Three 'elemental' diets were used in this study; two commercial diets - 'Flexical' (supplied by Mead-Johnson, Bristol Laboratories, Langley, Slough, U.K.) and 'Vivonex-HN' (supplied by Eaton Laboratories, Woking, Surrey, U.K.) - and one home-made diet, Diet B. The compositions of these diets are shown in Tables 10.2, 10.3 and 10.4 respectively. 'Flexical' and Diet B were similar in composition (see Table 10.5); the main difference was the higher casein hydrolysate content (20% of the calories) of Diet B compared with 9% of

Table 10.5 Comparison of the composition of 'Flexical' and Diet B

Component	<u>% of total calories</u>	
	<u>'Flexical'</u>	<u>Diet B</u>
Casein	9.0	20.6
Carbohydrate	61.0	54.0
Fat	30.0	25.4

the calories in 'Flexical'. Diet B was formulated by Bounous *et al* (1971a) in accordance with the nutritional requirements of rats (see Warner, 1952) and 'Flexical' was developed from Diet B for human use by reduction of the casein hydrolysate content to produce a diet more palatable for humans. 'Vivonex-HN' was included as a comparison; the amino nitrogen content is similar to that of Diet B but is present as free amino acids only (Table 10.3). The calorific values of the diets are shown in Table 10.6.

Table 10.2 Composition of 'Flexical'

Approximate Analysis (%w/w)

Protein Equivalent	9.9
Fat	15.0
Carbohydrate	67.8
Ash	2.7
Moisture	1.5

Nutrient Values

Each 454 grams of FLEXICAL powder when reconstituted with 1656 ml. water supplies approximately 2000 kilocalories in 2000 ml. Each 2000 kilocal. provides:

Protein Equivalent	45.0 g
Fat	68.0 g
Carbohydrate	308.0 g

Caloric Distribution

	g./2000 kilocal.	kilocal. /2000 kilocal.
Protein equivalent (9.0%)	45.0	180
Fat (30.0%)	68.0	602
Soy oil ¹	53.4	480
MCT	13.2	109
Soy lecithin	1.4	13
Carbohydrate (61.0%)	308.0	1218
Sucrose	201.8	807
Dextrin	96.8	387
Citrate	9.4	24

¹Nonhydrogenated

Amino Acids (gram per 2000 kilocal.)

Amino Acid	From Protein Hydrolysate	Added	Total
Arginine	1.87		1.87
Histidine	1.41		1.41
Isoleucine	2.85		2.85
Leucine	4.82		4.82
Lysine	4.03		4.03
Methionine	1.46	0.66	2.12
Cystine	0.20 ¹		
Total Sulphur Amino Acids			2.32
Phenylalanine	2.24		2.24
Tyrosine	0.65 ¹	0.53	1.18
Total Aromatic Amino Acids			3.42
Threonine	2.19		2.19
Tryptophan	0.49	0.22	0.71
Valine	3.54		3.54
Alanine	1.69		1.69
Aspartic acid	3.79		3.79
Glutamic acid	10.97		10.97
Glycine	1.12		1.12
Proline	5.11		5.11
Serine	2.98		2.98

¹Microbiological assay.

Vitamins and Minerals (per 2000 kilocal.)

Vitamin A, mg	1.72
Vitamin D, mg	0.01
Vitamin E, i.u.	45.
Vitamin C (ascorbic acid) mg	300.
Vitamin B ₁ (thiamine), mg	3.8
Vitamin B ₂ (riboflavin), mg	4.3
Vitamin B ₆ (pyridoxine), mg	5.
Vitamin B ₁₂ , mcg	15.
Niacin, mg	50.
Folic acid, mcg	400.
Choline, mg	500.
Biotin, mcg	300.
Vitamin K ₁ , mcg	250.
Calcium, g	1.2
Phosphorus, g	1.
Sodium, g	0.7
Potassium, g	2.5
Iron, mg	18.
Magnesium, mg	400.
Zinc, mg	20.
Manganese, mg	5.
Copper, mg	2.
Iodine, mcg	150.
Chloride, g	2.

Fatty acid

The approximate percentages of fatty acids present in FLEXICAL when prepared according to directions are as follows:

Fatty Acid	Grams per 2000 kilocal.	Percent of Total Fatty Acids
Octanoic (C ₈)	10.9	16.1
Decanoic (C ₁₀)	3.9	5.7
Lauric (C ₁₂)	—	tr
Myristic (C ₁₄)	—	tr
Palmitic (C ₁₆)	6.2	9.1
Stearic (C ₁₈)	2.1	3.1
Oleic (C ₁₈)	21.7	32.0
Linoleic (C _{18:2})	18.0	26.5
Linolenic (C _{18:3})	4.8	7.0

Information reproduced from Mead-Johnson product information booklet

Nutritional Information

Contents of one packet supply 300 kilocalories (1.256 MJ) and the following:—

		% by weight
Available nitrogen in the form of pure amino acids (amino acid content 12.93g)	2.00g	2.5
Fat as highly purified safflower oil (80% triglyceride of linoleic acid)	0.261g	0.326
Carbohydrate as glucose solids	63.1g	78.9

Caloric contribution:

Amino acids	18.26%
Fat	0.78%
Carbohydrate	80.96%

Ten packets of Vivonex HN supply 20.0g of available nitrogen, 2.61g fat, 631g carbohydrate and the following vitamins, amino acids and minerals:—

Amino Acids

ESSENTIAL AMINO ACIDS	% Total Amino Acids	g/10 packets
L-Isoleucine	4.10	5.30
L-Leucine	6.48	8.38
L-Lysine	4.87	6.30
L-Methionine	4.19	5.42
L-Phenylalanine	7.01	9.05
L-Threonine	4.10	5.30
L-Tryptophan	1.27	1.64
L-Valine	4.52	5.84

Total essential amino acids: 36.54% 47.25

NON-ESSENTIAL AMINO ACIDS	% Total Amino Acids	g/10 packets
L-Alanine	5.11	6.61
L-Arginine	3.99	5.16
L-Aspartic Acid	10.90	14.09
L-Glutamine	17.97	23.24
Glycine	9.70	12.54
L-Histidine	2.32	3.00
L-Proline	6.82	8.82
L-Serine	4.09	5.29
L-Tyrosine	2.56	3.31

Total non-essential amino acids: 63.46% 82.05

Electrolytes

Cations	In normal dilution—		In powder form	
	80g in 300 ml m-equiv/100 ml	mg/80g packet	mg/100g	mg/10 packets
Sodium	3.35	231.3	289	2313
Potassium	1.79	210.5	263	2105
Calcium	1.33	80.0	100	800
Magnesium	0.958	35.0	43.75	350
Manganese	0.00340	0.281	0.35	2.81
Iron	0.0119	1.00	1.25	10.0
Copper	0.00203	0.194	0.24	1.94
Zinc	0.0127	1.25	1.56	12.5
Anions	m-equiv/100 ml		mg/100g	
			mg/100g	mg/10 packets
Chloride	5.24	557.3	696.6	5573
Phosphate (as P)	2.58*	80.0	100	800
Acetate	0.0211	3.74	4.68	37.4
Sulphate (as S)	0.0239†	1.15	1.44	11.5
Iodide	0.000038	0.0144	0.02	0.144
Sorbate	0.268	89.2	111.5	892

Ten packets also contain 0.22 µg Cobalt as Vitamin B₁₂

Vitamins

	Per 80g packet	Per 100g	In ten 80g packets
Vitamin A (Retinol) µg	150	187.5	1500
Vitamin D ₂ (Ergocalciferol) µg	1.0	1.25	10
Vitamin E I.U.	3.0	3.75	30
Vitamin K ₁ µg	6.7	8.38	67
d-Biotin mg	0.02	0.03	0.2
Choline bitartrate mg	15.4	19.25	154
Vitamin B ₁₂ µg	0.5	0.63	5
Folic acid mg	0.01	0.01	0.1
Inositol mg	11.65	14.56	116.5
Nicotinamide mg	1.33	1.66	13.3
d-Calcium pantothenate mg	1.0	1.25	10
Pyridoxine HCl mg	0.2	0.25	2
Vitamin B ₁ (Thiamine) mg	0.12	0.15	1.2
Vitamin B ₂ (Riboflavin) mg	0.12	0.15	1.2
Vitamin C (Ascorbic acid) mg	7.0	8.75	70

Information reproduced from Eaton Laboratories product information sheet

Table 10.4 Composition of Diet B

<u>Component</u>	<u>Source</u>	<u>Amount (g/100 g)</u>
Casein hydrolysate (enzymic)	Sigma London Chemical Co. Ltd.	24.9
Sucrose (Grade 11)	Sigma London Chemical Co. Ltd.	44.7
Medium chain triglyceride	Cow & Gate Ltd. Trowbridge, Wilts.	6.9
Corn oil	Ma zola	4.3
Olive oil	Pacchini Oil Importing Co. Ltd, Glasgow	10.4
Cod liver oil	British Cod Liver Oils Ltd, Marfleet, Hull	1.3
Vitamin mixture	United States Biochemical Corporation	2.5
Jones Foster* salt mixture	United States Biochemical Corporation	5.0

* Altman and Dittmer (1972)

Table 10.6 The calorific values of the diets

<u>Diet</u>	<u>Calorific value (kJ/g)</u>
'Flexical'	1.06
Diet B	1.06
'Vivonex-HN'	0.90
'Oxoid Diet 86'	0.64

The diets were presented in dry form in glass bowls in the cages of the rats. In some instances flavouring (supplied by Eaton Laboratories for use with 'Vivonex-HN') was added to Diet B (1 sachet/100 g of diet).

Animals

The rats were caged individually or in groups of 4 and fed on an 'elemental' diet for 1, 2 or 6 weeks prior to use and the diet was continued after 5-fluorouracil administration. Body weights and food intakes were recorded daily.

5-fluorouracil administration

Rats were weighed and 5-fluorouracil was administered under light ether anaesthesia at a mean dose of 1.44 mmol/kg body weight. In view of the different systemic toxicities of the two salts of 5-fluorouracil (discussed in Chapter 8) both the Tris and the sodium salts of 5-fluorouracil were used.

Water absorption

Water absorption rates by the whole jejunum plus ileum were

estimated in vitro by the method described in Chapter 3 in control (uninjected) rats and on the first and third days after 5-fluorouracil administration.

Peptide hydrolase activities and DNA estimation

Mucosal DNA contents and cytoplasmic peptide hydrolase activities against three dipeptide substrates - Gly-L-Met, L-Leu-Gly and L-Val-L-Leu - were estimated in the whole jejunum plus ileum from control (uninjected) rats and on the first and third days after 5-fluorouracil administration. The methods were those described in Chapters 5 and 4 respectively.

Proline absorption

Proline absorption rates were estimated in vitro in the perfused small intestine by addition of about 1 mmol proline/l (Sigma London Chemical Co.) to the perfusate; proline was estimated in the serosal secretion collected over a 15 minute period commencing 45 minutes after the start of perfusion. The composition of the secretion reaches a steady state during this period and the appearance of proline in the serosal secretion is equivalent to net absorption or transport if measured under steady state conditions (Fisher and Gardner, 1974; Gardner, 1976).

Proline estimation

The serosal secretion was deproteinised by addition of solid sulphosalicylic acid (30 mg/ml) and after 20 minutes samples were centrifuged for 20 minutes at 4000 g at room temperature. Proline was estimated in the supernatant and in the perfusate on a Locarte

floor model Mark IV amino acid analyser. Details of the amino acid analysis programme, by stepwise elution with sodium citrate buffers on a single column, were given by Gardner (1981)

Elliot and Gardner (1976) described a method for the estimation of proline in the presence of amino acids which used isatin as a chromogen. It was hoped that this method could be used for the rapid determination of proline in the serosal secretion. However, whilst the method was satisfactory for the estimation of proline dissolved in citrate buffer (0.1 mol/l) or in the perfusate in the absence of glucose, addition of glucose prevented development of the blue complex between proline and isatin. The method was therefore unsuitable for the estimation of proline in the serosal secretion.

Table 10.7 Effects of 'elemental' diets on growth rates and food
intakes of male and female rats

<u>Females</u>				
Diet	Food intake		Weight gain	
	(g/day/rat)	(kJ/day/rat)	(g/day/rat)	(mg/day/kJ)
'Oxoid Diet 86'	16.8 \pm 0.41 (12)	188.5	2.46 \pm 0.24 (8)	13.1
'Vivonex-HN'	15.7 \pm 0.60 (12)	246.5	1.71 \pm 0.29 (8)	6.9
'Flexical'	14.8 \pm 0.62 (12)	273.0	2.09 \pm 0.19 (8)	7.7
Diet B	11.3 \pm 0.50 (12)	208.2	1.52 \pm 0.43 (8)	7.3
Diet B + Tomato flavour	13.7 \pm 0.51 (12)	252.4	1.99 \pm 0.34 (8)	7.9
<u>Males</u>				
'Oxoid Diet 86'	20.2 \pm 0.31 (32)	226.6	6.05 \pm 0.21 (34)	26.7
Diet B	16.3 \pm 0.30 (78)	300.3	5.31 \pm 0.35 (32)	17.7
Diet B + Tomato flavour	17.4 \pm 0.30 (70)	320.5	5.63 \pm 0.21 (30)	17.6
Diet B + Tomato flavour, 1rat/cage	19.2 \pm 0.63 (48)*	353.7	6.50 \pm 0.36 (8)	18.4

Numbers of observations are shown in parentheses. Those for food intakes refer to observations on groups of 4 rats (excluding *) and those for body weights refer to individual rats, observed over a 5 day period.

RESULTS

CONTROL EXPERIMENTS ON RATS NOT INJECTED WITH 5-FLUOROURACIL

Growth rates and food intakes of rats fed on 'elemental' diets

All female rats fed on an 'elemental' diet lost weight, varying from 5 - 15 g per rat, over the first two days on the diet. This was associated with a lower food intake, by 40 - 50%, than was observed subsequently. This phenomenon was less apparent in male rats in which the maximum body weight loss was 8 g and in most instances the food intake over the first 24 hours on the diet was only 20% less than that observed subsequently. The first two days on the 'elemental' diet have therefore been excluded from the estimates of food intakes and growth rates since this period probably represents adaptation to a change of diet.

Table 10.7 shows the daily body weight gains and food intakes of both male and female rats fed for 1 week on the standard diet, Oxoid Diet 86, or on one of three 'elemental' diets. The weight gain is also expressed per kJ food intake. Daily food intakes and growth rates of both male and female rats were reduced (by 5 - 38% depending on the diet) when the standard diet was replaced by an 'elemental' diet; the weight gain per kJ intake was also reduced, by about 43% in females and 33% in males, although the total energy intake was increased by between 11% and 45% in female rats and between 33% and 56% in male rats. Both the growth rates and food intakes of male rats were greater than those of female rats; growth rates were 46% greater and food intakes were 20% greater in male than in female rats. Also the weight gain per kJ intake was greater by a factor of

2 in male than in female rats.

Effect of flavouring the diet on food intakes and growth rates

Since food intakes and growth rates were reduced in rats fed on an 'elemental' diet, an attempt was made to increase the spontaneous intake of food by the addition of flavouring to the diet. The flavours used were those supplied by Eaton Laboratories for addition to unflavoured 'Vivonex-HN' and they were added to Diet B (1 sachet/100 g diet). Two male rats were caged individually and given 4 bowls of Diet B, each with a different flavour added. The position of the bowls in the cages was varied daily and the food intake from each bowl was recorded over the next 7 days. The results are shown in Table 10.8. Both rats ate a much greater amount of 'Tomato' flavoured diet than of the other three flavours.

Subsequently, when both male and female rats were fed solely on 'Tomato flavoured Diet B' the food intakes were greater by 21% and 7% respectively, and growth rates were greater by 21% and 5% respectively, than those observed in rats fed on unflavoured Diet B (Table 10.7). When male rats were caged individually and fed on 'Tomato flavoured Diet B' the growth rate was slightly greater, by 7%, than those observed in rats fed on the Oxoid diet.

Gross appearance of intestines from rats fed on 'elemental' diets

Intestines from rats fed on 'Vivonex-HN' were similar in gross appearance to those from rats fed on the standard diet, Oxoid Diet 86. In contrast, those from rats fed on 'Flexical' or on Diet B were very pale in colour and there was a large deposit of fat around the mesentery. The serosal secretion from perfused intestines

Table 10.9 Intestinal water absorption rates and dry weights in rats fed for 1, 2 or 6 weeks on 'elemental' diets

Diet	Sex	Time on diet (weeks)	Water absorption rate (μl/cm/hr)	Dry weight (mg/cm)
'Oxoid Diet 86'	F	-	138.7 \pm 9.0 (13)	11.5 \pm 0.37 (10)
'Flexical'	F	1	154.6 \pm 8.6 (9) N.S.	14.8 \pm 0.77 (8) **
'Flexical'	F	2	197.3 \pm 24.4 (4) **	!
'Vivonex -HN'	F	1	190.1 \pm 10.5 (12) **	10.3 \pm 0.42 (12)@
'Vivonex -HN'	F	6	184.2 \pm 11.3 (5) **	10.6 \pm 0.37 (5) N.S.
Diet B	F	1	164.5 \pm 10.9 (7) N.S.	13.8 \pm 0.41 (8) **
'Oxoid Diet 86'	M	-	138.6 \pm 8.9 (9)	13.7 \pm 0.83 (9)
Diet B	M	1	165.4 \pm 11.4 (7) N.S.	15.9 \pm 1.17 (8) **

Values are the mean \pm S.E.M. and the number of observations is shown in parentheses. The significance levels of the differences from the values in Oxoid-fed animals are indicated thus:

** $P < 0.02$, N.S. $P > 0.1$.

@ The values for rats fed for 1 week on 'Vivonex-HN' are from Gardner and Heading (1979).

! No estimate available.

removed from rats fed on 'Flexical' or Diet B was opaque, presumably a reflection of the high fat content of these diets.

Effect of 'elemental' diets on intestinal absorption, dry weights and peptide hydrolase activities

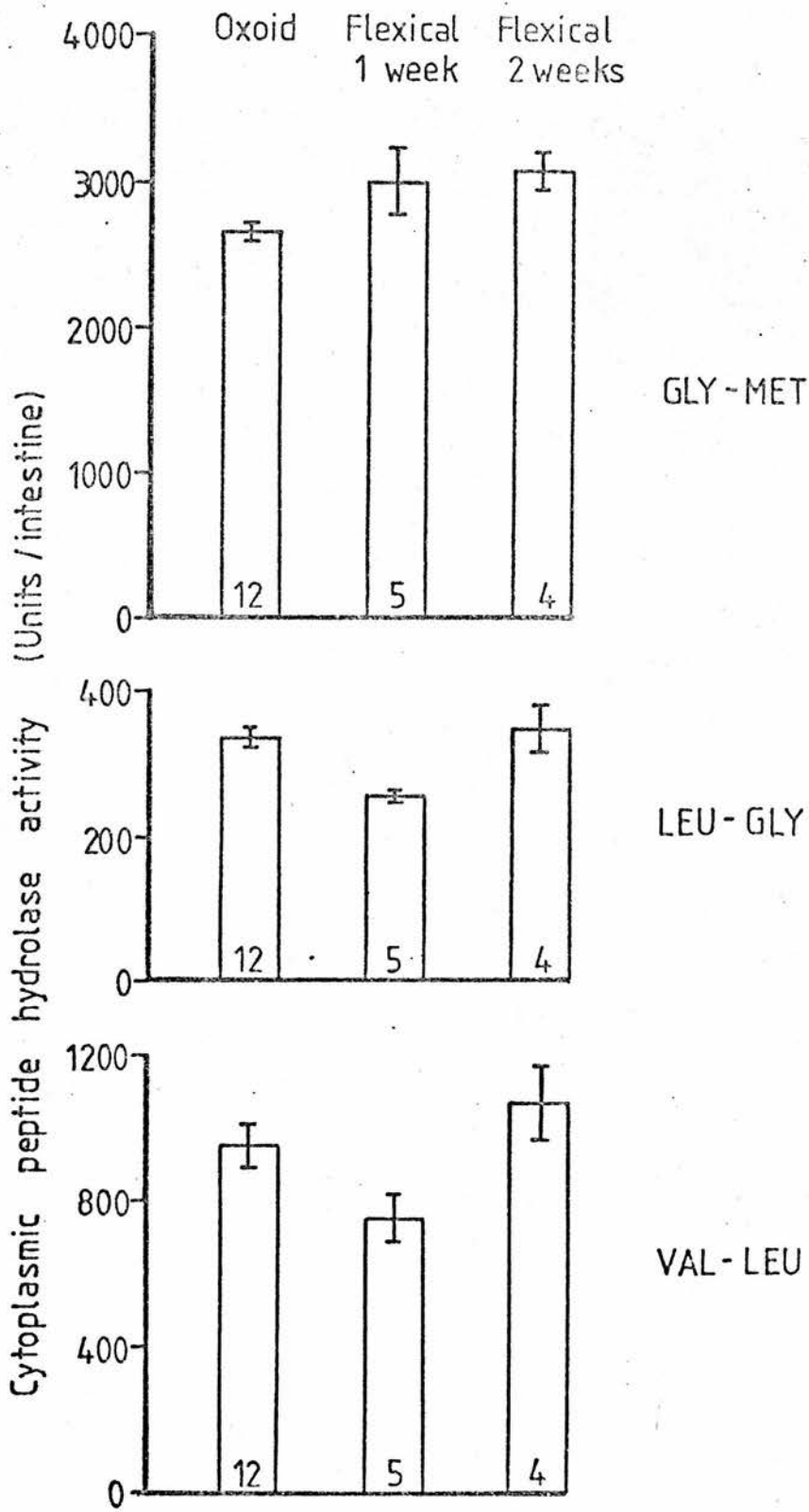
Table 10.9 shows the water absorption rates and dry weights of intestines from rats fed on the Oxoid diet and from rats fed on various 'elemental' diets for 1, 2 or 6 weeks previously. Water absorption rates were increased significantly ($P \leq 0.02$) in female rats fed for 2 weeks on 'Flexical' or for 1 (results from Gardner and Heading, 1979) and 6 weeks on 'Vivonex-HN' when compared with those from rats fed on the Oxoid diet. Although absorption rates were increased in both male and female rats fed for 1 week on Diet B and in female rats fed for 1 week on 'Flexical', this increase was not significant ($P > 0.1$). The intestinal dry weights were significantly increased in rats fed on 'Flexical' or Diet B ($P \leq 0.02$).

The cytoplasmic peptide hydrolase activities in intestines from rats fed for 1 or 2 weeks on 'Flexical' were not significantly different ($P > 0.1$) from those in intestines from rats fed on the Oxoid diet (Fig. 10.1).

Absorption rates in male and female rats

The similarity between water absorption rates estimated on intestines from male and female rats has already been discussed (Chapter 8). This similarity is also apparent in rats fed on Diet B (Table 10.9).

Fig. 10.1 Cytoplasmic peptide hydrolase activities in the mucosa of the small intestine from rats fed on the Oxoid diet or for 1 or 2 weeks on 'Flexical'. Substrates were Gly-L-Met, L-Leu-Gly and L-Val-L-Leu. Values are the mean \pm S.E.M. and the number of animals is shown within the bars.



EXPERIMENTS ON RATS INJECTED WITH 5-FLUOROURACIL

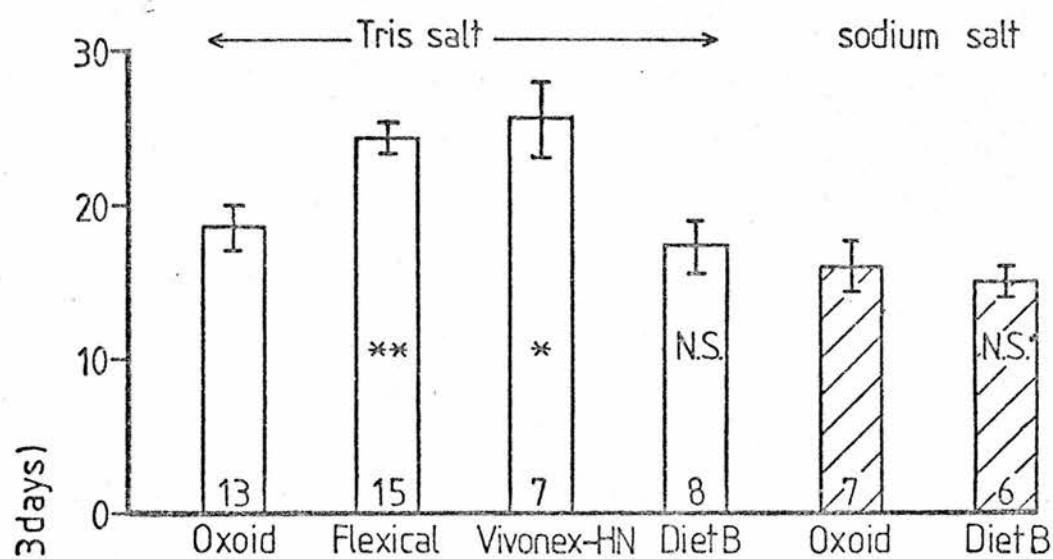
After establishment of the basic physiological effects of 'elemental' diets on the intestine experiments were designed to test whether these diets protect the intestine from the toxic effects of 5-fluorouracil. The following parameters were used as indices of 5-fluorouracil toxicity: food intake, body weight loss, mucosal DNA content, peptide hydrolase activity, intestinal water absorption and dry weight, and the incidence of diarrhoea.

Body weight losses and food intakes during the first 3 days after 5-fluorouracil administration

Body weights and food intakes are both severely reduced during the first 3 days after administration of 5-fluorouracil (see Chapter 8). Figs. 10.2 and 10.3 show the total body weight losses and food intakes respectively during the first 3 days after 5-fluorouracil (Tris and sodium salts) administration in both female (Figs. 10.2A and 10.3A) and male (Figs. 10.2B and 10.3B) rats; the rats were fed either on the Oxoid diet or on an 'elemental' diet ('Flexical', 'Vivonex-HN' or Diet B with Tomato flavouring added) for at least 1 week prior to drug administration. Body weight losses were significantly increased in female rats fed on 'Flexical' ($P < 0.01$) or 'Vivonex-HN' ($P < 0.02$) prior to 5-fluorouracil administration; the food intake of these rats was also decreased significantly ($P < 0.05$). In contrast, body weight losses and food intakes were similar when rats were fed on the Oxoid diet or Diet B. Thus if body weight loss is regarded as a criterion of toxicity, then the two commercial 'elemental' diets increase the toxicity of 5-fluorouracil.

Fig. 10.2 Total body weight loss during the first 3 days after administration of 5-fluorouracil (Tris or sodium salt) in female (A) and in male (B) rats. The rats had been fed on the Oxoid diet or on various 'elemental' diets for at least 1 week before 5-fluorouracil administration and thereafter. Values are the mean \pm S.E.M. and the number of animals is shown within the bars. The significance levels of the differences from values in 'control' rats fed on the Oxoid diet and injected with the same salt of 5-fluorouracil are indicated thus: ** $P \leq 0.01$, * $P \leq 0.02$, N.S. $P > 0.1$.

(A) FEMALES



(B) MALES

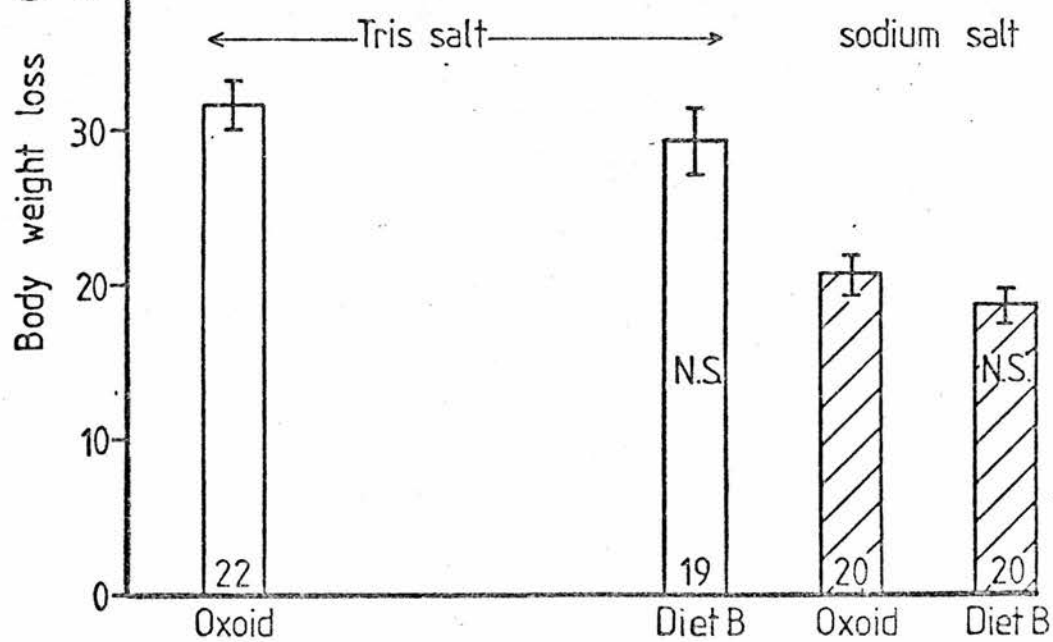
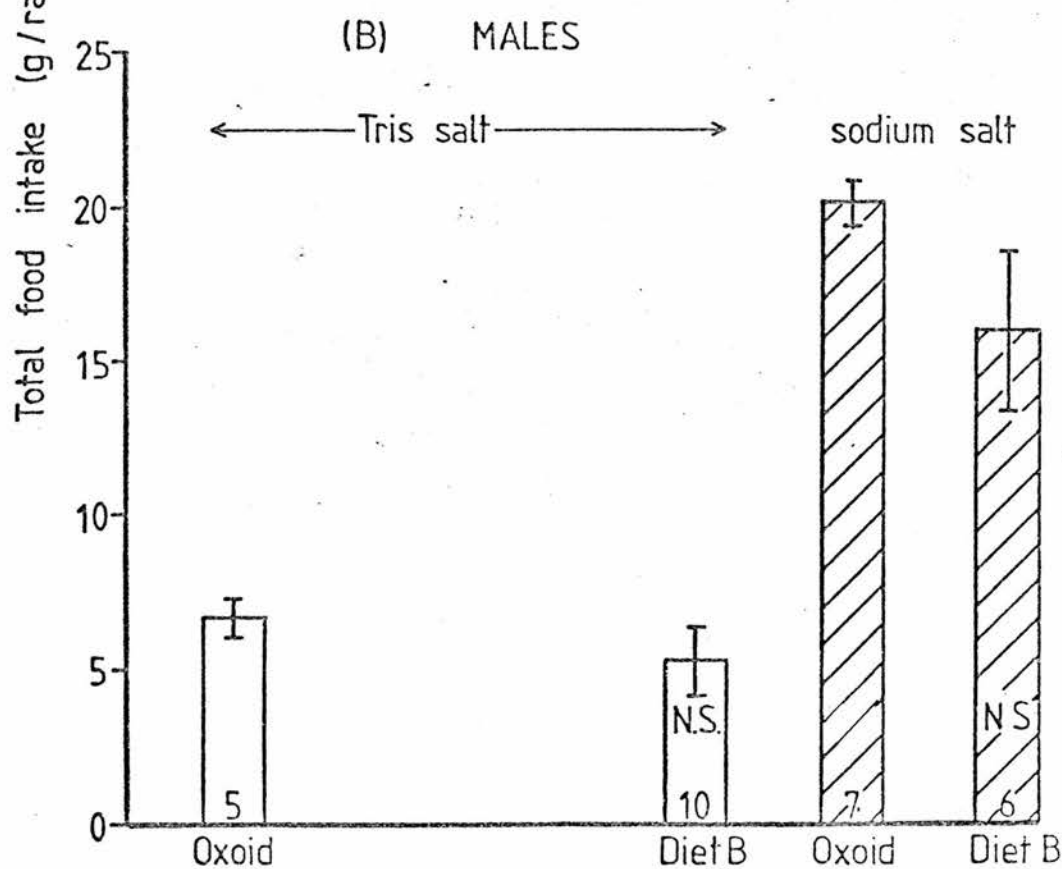
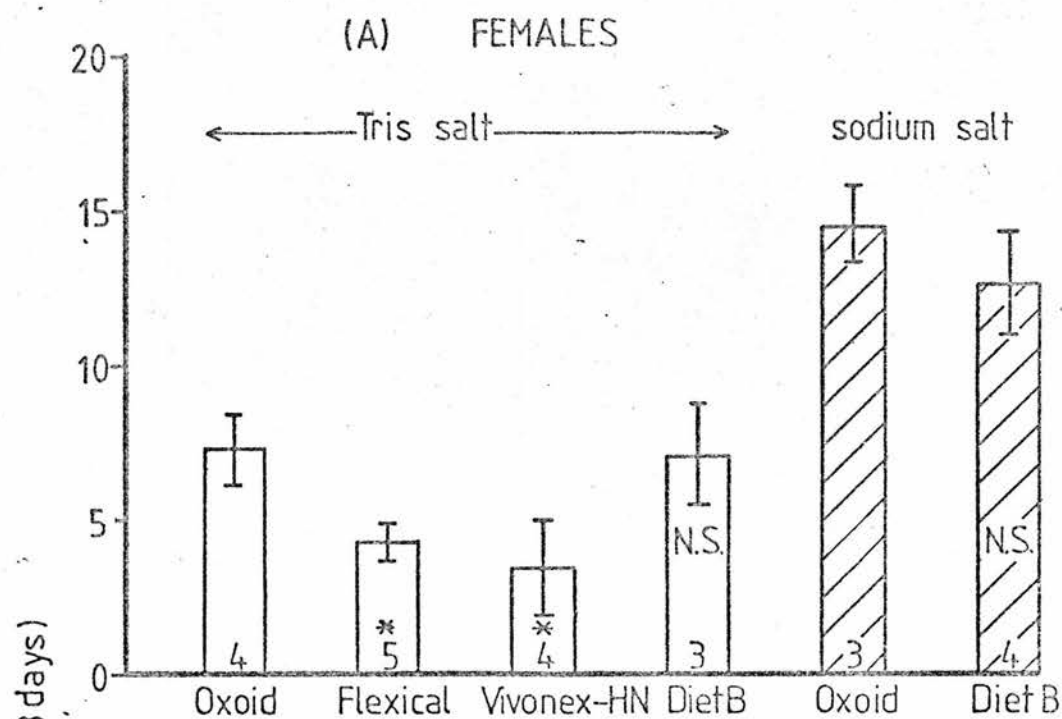


Fig. 10.3 Total food intakes during the first 3 days after administration of 5-fluorouracil (Tris or sodium salt) in female (A) and in male (B) rats. The rats had been fed on the Oxoid diet or on various 'elemental' diets for at least 1 week before 5-fluorouracil administration and thereafter. Values are the mean \pm S.E.M. of observations on groups of 4 rats and the number of observations is shown within the bars. The significance levels of the differences from values in rats fed on the Oxoid diet and injected with the same salt of 5-fluorouracil are indicated thus: * $P < 0.02$, N.S. $P > 0.1$.



The effects of the Tris salt of 5-fluorouracil on body weights and food intakes are greater than those of the sodium salt of 5-fluorouracil (see Chapter 8). This is also demonstrated in Figs. 10.2 and 10.3; body weight losses were significantly less ($P < 0.02$) in rats, fed on the Oxoid diet or Diet B, injected with the sodium salt of 5-fluorouracil, when compared with those injected with the Tris salt of 5-fluorouracil. Similarly, the food intake during the first 3 days after administration of the sodium salt was significantly greater ($P < 0.001$) than after administration of the Tris salt of 5-fluorouracil.

Mucosal DNA content and cytoplasmic peptide hydrolase activities after 5-fluorouracil administration

Mucosal cytoplasmic peptide hydrolase activities and DNA contents were estimated in control (uninjected) female rats and in female rats that had been injected with 5-fluorouracil (Tris salt) 1 or 3 days previously. The rats were fed on the Oxoid diet or 'Flexical' for at least 7 days prior to and then after injection. The results are shown in Figs. 10.4 and 10.5. There was a significant decrease in the mucosal DNA content both 1 and 3 days after 5-fluorouracil administration, to about 47% and 38% of the normal content respectively ($P < 0.001$) and the decrease was similar for both diets (Fig. 10.4). Cytoplasmic peptide hydrolase activities were unaffected 1 day after 5-fluorouracil administration but were decreased to between 36 and 71% of normal activities by the third day after administration ($P < 0.01$) depending upon the activity measured (Fig. 10.5). This pattern was observed for all three activities measured and for both diets.

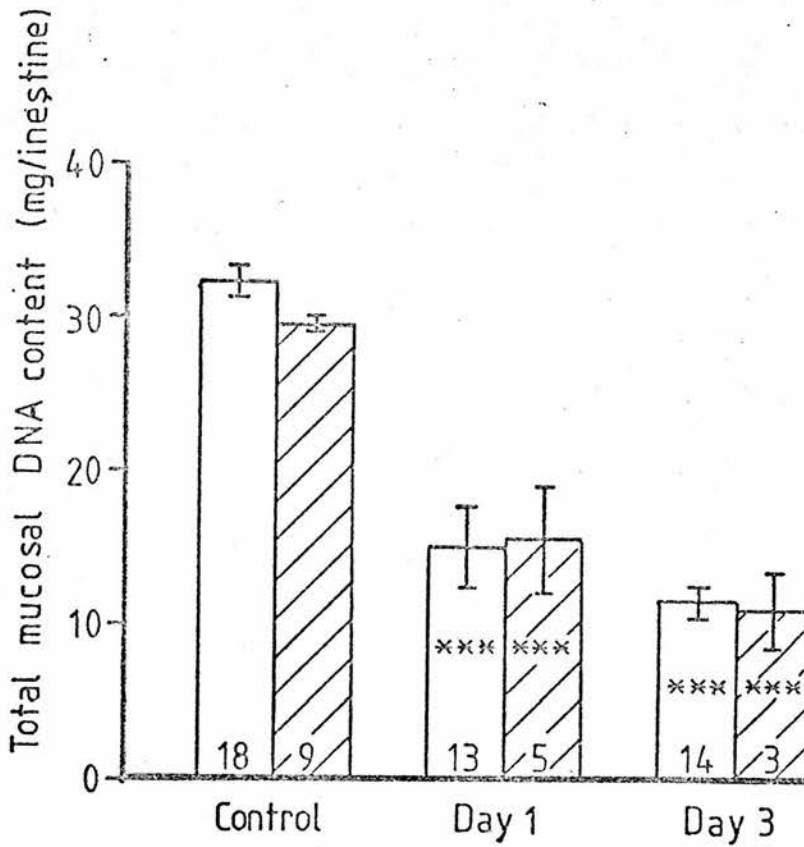


Fig. 10.4 DNA content of the mucosa from the whole jejunum plus ileum from control female rats and on the first and third days after administration of the Tris salt of 5-fluorouracil. Rats were fed (open bars) (hatched bars) either on the Oxoid diet or on 'Flexical' for 1 week before and after 5-fluorouracil injection. Values are the mean \pm S.E.M. and the number of animals is shown within the bars. The significance levels of the differences from the respective controls are indicated thus: *** $P < 0.001$

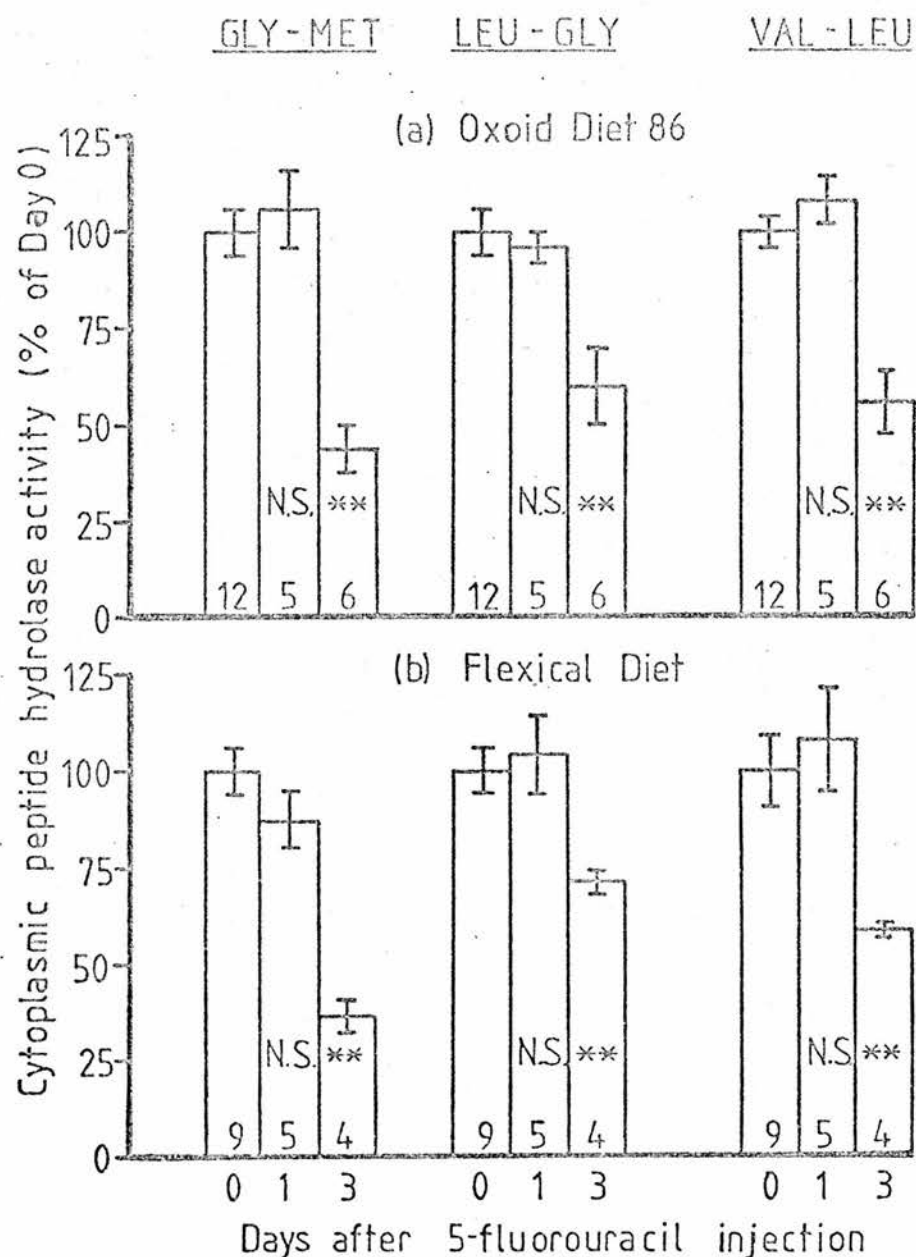


Fig. 10.5 Cytoplasmic peptide hydrolase activities in the mucosa of the small intestine from control female rats, fed on the Oxoid diet or on Diet B, and on the first and third days after 5-fluorouracil (Tris salt) administration in female rats fed on the Oxoid diet or 'Flexical'. Substrates were Gly-L-Met, L-Leu-Gly and L-Val-L-Leu. Values are the mean \pm S.E.M. and the number of animals is shown within the bars. The significance levels of the differences from the respective controls are indicated thus: ** $P < 0.01$, N.S. $P > 0.1$.

Intestinal absorption, dry weights and the incidence of diarrhoea in rats fed on an 'elemental' diet before and after 5-fluorouracil administration

Fig. 10.6 shows the intestinal water absorption rates and dry weights on the third day after 5-fluorouracil administration in male (hatched bars) and female (open bars) rats fed for 1, 2 or 6 weeks previously on one of three 'elemental' diets. The results from rats fed on the Oxoid diet are included for comparison and the results from experiments with unflavoured and flavoured Diet B have been combined since they were very similar. Water absorption rates on the third day after 5-fluorouracil administration are similar regardless of the nature of the diet. This is true both in male and female rats and for both the Tris and sodium salts of 5-fluorouracil. However, intestinal dry weights on the third day after administration of 5-fluorouracil to rats fed on Diet B were significantly greater ($P < 0.05$) than those from rats fed on the Oxoid diet. Table 10.10 shows the incidence of diarrhoea in these same animals (the number that developed diarrhoea is expressed as a percentage of all rats injected) during the first 3 days following 5-fluorouracil injection. The incidence was very similar for all diets except in the case of Diet B when there was a marked reduction in the incidence of diarrhoea in both male and female rats. Diarrhoea was not observed in control (uninjected) rats fed on any of the 'elemental' diets or on the Oxoid diet.

Correlation between intestinal absorption and body weights

Intestinal water absorption rates were 54% greater on the third day after 5-fluorouracil (Tris salt) administration in rats fed on

Fig. 10.6 Intestinal water absorption rates and dry weights on the third day after 5-fluorouracil (Tris or sodium salt) administration in male (hatched bars) and female (open bars) rats; rats were fed on the Oxoid diet or on one of 3 'elemental' diets for various times before and then after injection. Values are the mean [±] S.E.M. and the number of animals is shown within the bars.

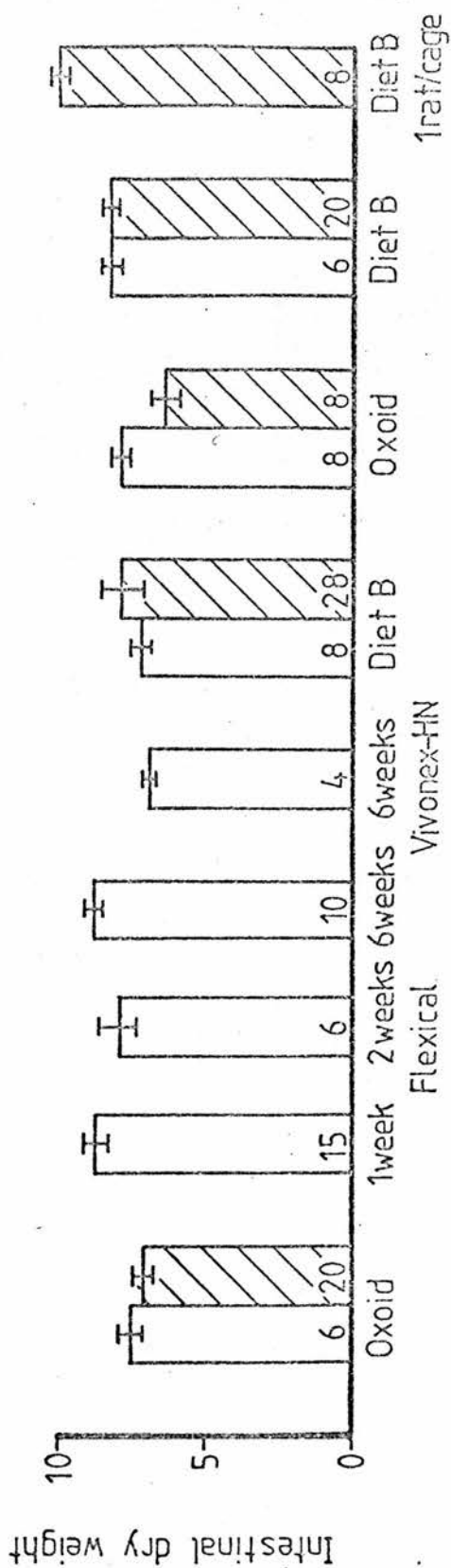
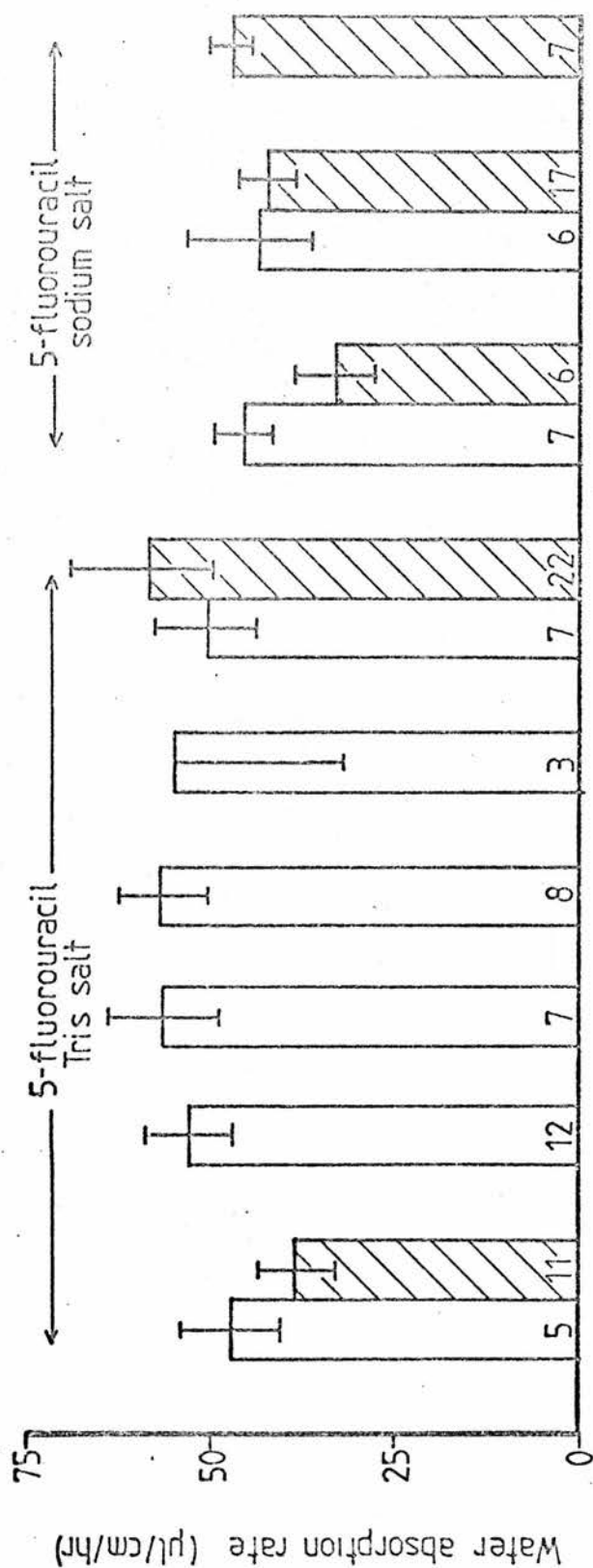


Table 10.10 The incidence of diarrhoea 3 days after administration
of the Tris or sodium salt of 5-fluorouracil in male and female rats
fed on 'elemental' diets

Diet	Time on diet (weeks)	<u>Incidence of diarrhoea (% of total)</u>			
		Tris salt		Sodium salt	
		female	male	female	male
'Oxoid Diet 86'	-	83.3 (6)	80.0 (20)	87.5 (8)	87.5 (8)
'Flexical'	1	69.6 (15)	-	-	-
'Flexical'	2	72.7 (6)	-	-	-
'Flexical'	6	70.0 (10)	-	-	-
'Vivonex-HN'	6	100.0 (4)	-	-	-
Diet B	1	37.5 (8)	28.6 (28)	37.5 (6)	30.0 (20)
Diet B (1rat/cage)	1	-	-	-	25.0 (8)

The number of animals is shown in parentheses and the values are the percentage of these animals that had diarrhoea.

- No estimate available.

Diet B than the corresponding rates from animals fed on the Oxoid diet, although this difference was not significant due to the large inter-animal variability ($P > 0.1$). Fig. 10.7 shows the distribution of absorption rates estimated on the third day after 5-fluorouracil administration (Tris and sodium salts) in male rats fed either on the Oxoid diet or on Diet B. It is clear from the results for individual rats that some animals benefited from this particular 'elemental' diet. The absorption rates estimated on intestines from rats fed on Diet B were distributed over a wider range than those from rats fed on the Oxoid diet; some were within the range of absorption rates observed in control (uninjected) Oxoid-fed rats. Therefore the individual histories of the rats fed on Diet B prior to 5-fluorouracil administration were studied closely, in order to attempt to identify any features in the animals 'history' (e.g. growth rate before 5-fluorouracil injection) which were correlated with a good prognosis. Water absorption rates on the third day after administration of the Tris salt of 5-fluorouracil in male rats fed on Diet B correlated positively with the body weight of the rats both on the day of injection (Fig. 10.8a, $P < 0.01$) and with the body weight on the third day after injection (Fig. 10.8b, $P < 0.001$). It should be stressed that the 5-fluorouracil dose was calculated for each rat so that it was proportional to body weight at time of injection. They did not correlate with the body weight at the start of dietary modification (Fig. 10.8c, $P > 0.1$). No such correlations were apparent for any other group in Fig. 10.6.

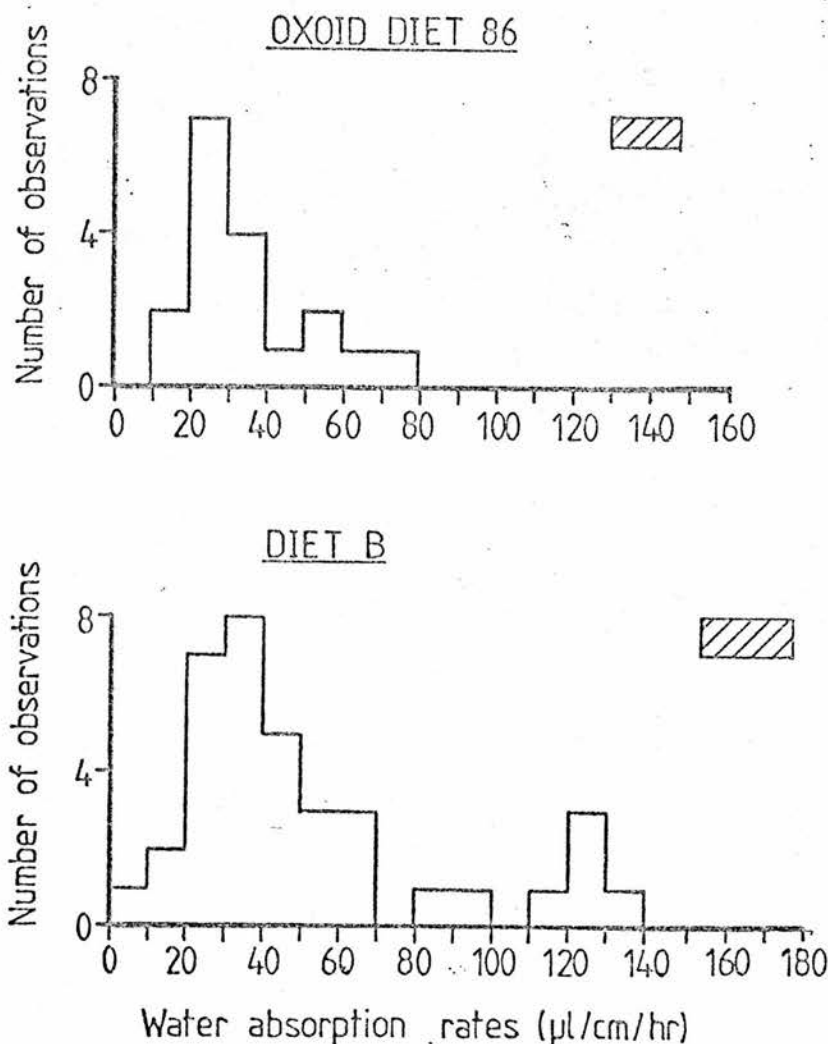


Fig. 10.7 Distribution of absorption rates measured on the third day after administration of 5-fluorouracil (Tris or sodium salt) to male rats fed on Diet B (flavoured or unflavoured) for at least 1 week before injection and thereafter. Those from rats fed on the Oxoid diet are shown for comparison. Also shown is the range (mean \pm S.E.M.) of absorption rates observed in uninjected male rats fed on the same diet (hatched bar).

Fig. 10.8 Intestinal water absorption rates 3 days after 5-fluorouracil (Tris salt) administration to male rats fed on Diet B (flavoured and unflavoured). Absorption rates, estimated on individual intestines, are plotted against body weight at the time of injection (a), body weight 3 days after 5-fluorouracil injection (b), and body weight at the start of dietary treatment (c). Also shown are the correlation coefficient (r), the significance level of the correlation (P), and the number of animals (n).

Fig. 10.8a

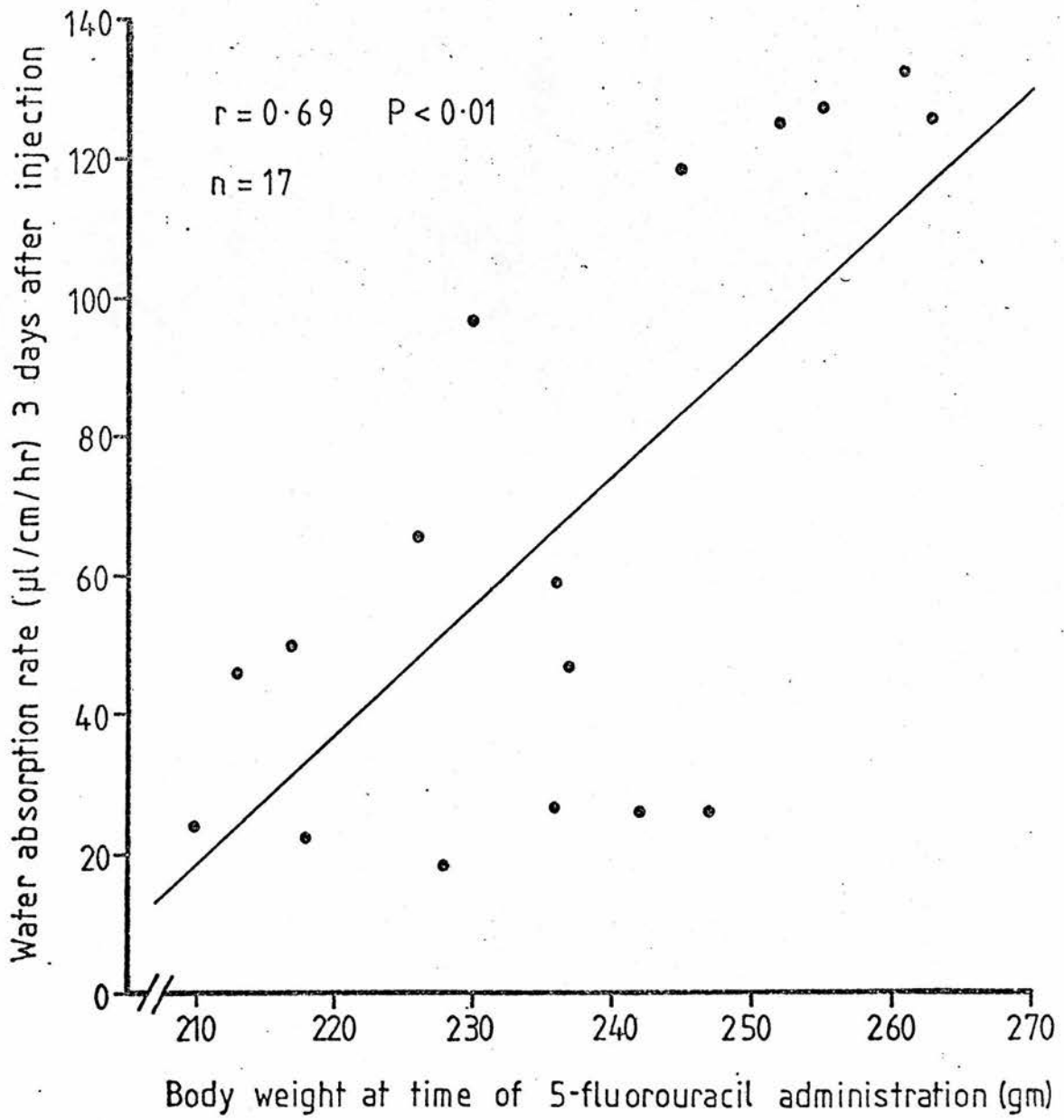


Fig.10.8b

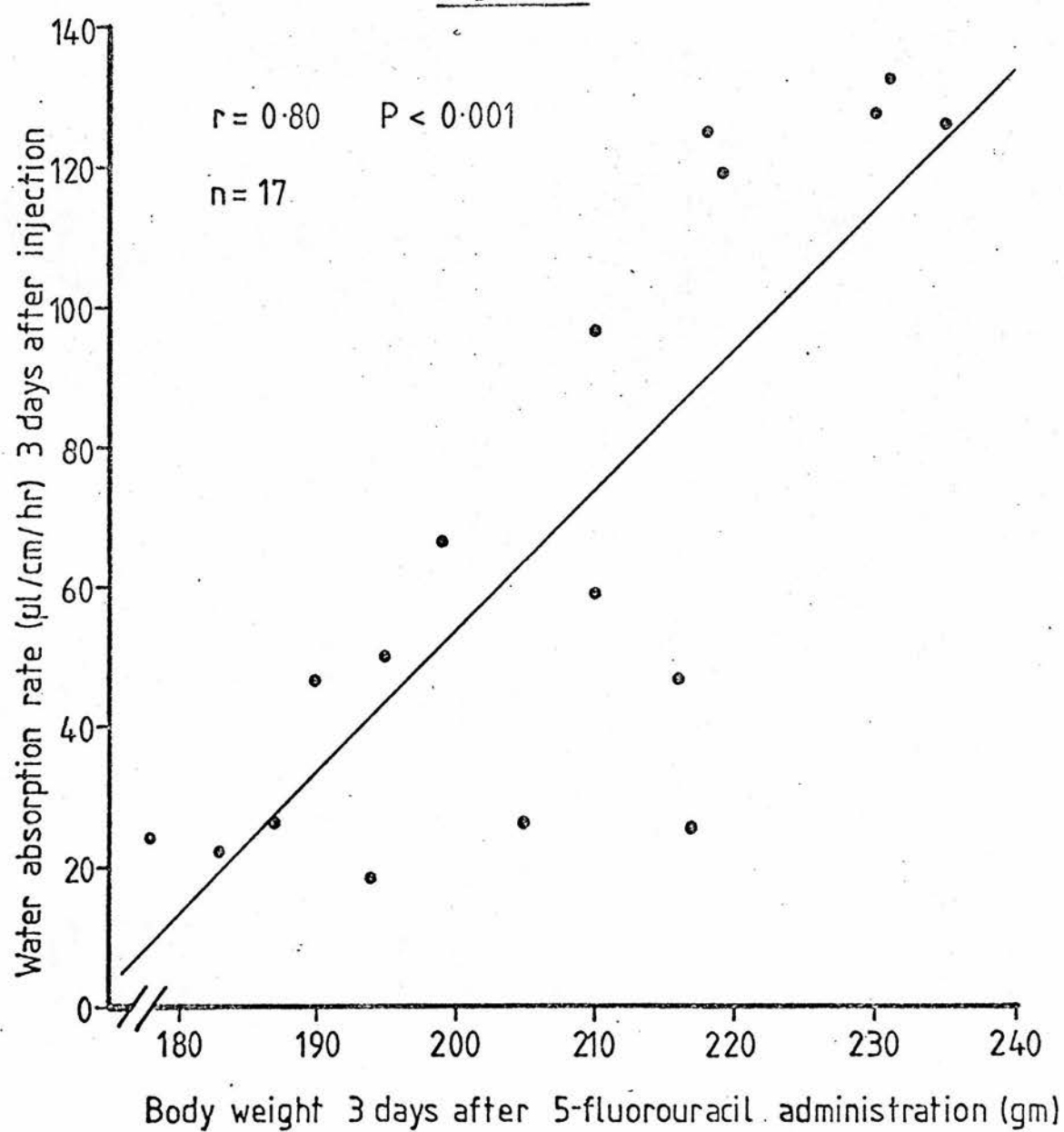
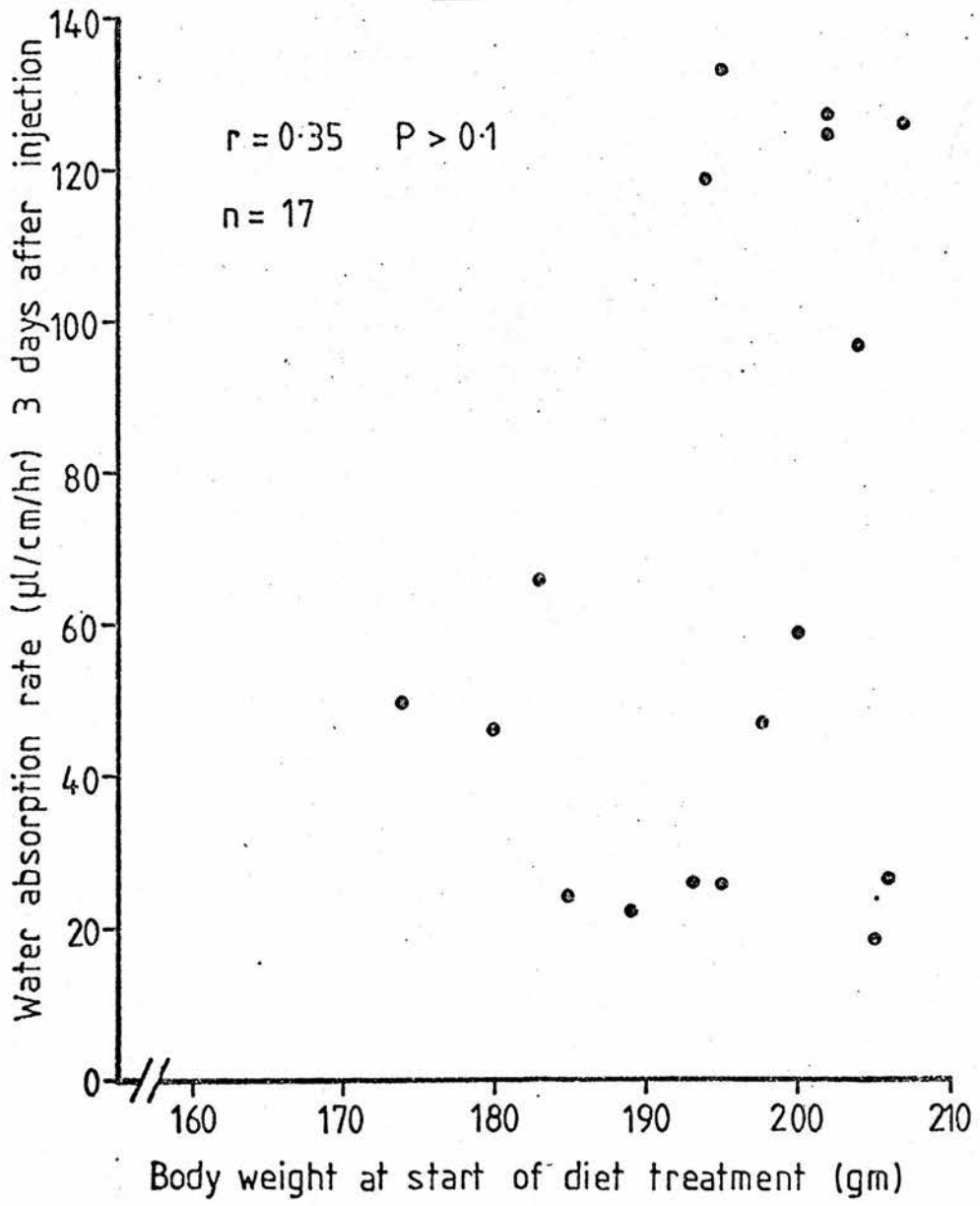


Fig. 10.8c



Effect of increased food intake and growth rate on 5-fluorouracil toxicity

The above observations suggest that the rats which benefited from the 'elemental' diet may have been those with the highest growth rates. Thus an attempt was made to increase the food intakes and growth rates of rats fed on Diet B. Both food intakes and growth rates were increased, by 18% and 22% respectively, when rats were caged individually and fed on Diet B with Tomato flavouring added (Table 10.7). However, water absorption rates on the third day after 5-fluorouracil administration were not significantly greater in these rats than those of rats fed on the Oxoid diet and caged in groups of 4 (Fig. 10.6). Hence the correlations observed between absorption rates on the third day after 5-fluorouracil administration and the body weight at the time of injection probably does not indicate a causal relationship and there is no benefit to be gained from increasing the food intake.

Effects of 5-fluorouracil on intestinal proline absorption in rats fed on an 'elemental' diet

Mohiuddin and Kramer (1978) reported that the intestinal absorption of proline, estimated in vitro, was decreased in rats following irradiation of the intestine in vivo and that the decrease could be reduced if rats were fed on an 'elemental' diet. Their's is the only independent evidence in favour of Bounous' theory; hence it was of importance to perform similar studies and, therefore, proline absorption rates were estimated on intestines from male rats 3 days after 5-fluorouracil administration. Rats were fed either on the Oxoid diet or on Diet B (Tomato flavoured) for 1 week before and

Table 10.11 Rates of proline transport across intestines from male rats fed for 10 days on Diet B and from male rats fed on the Oxoid diet or Diet B for 7 days before and for 3 days after 5-fluorouracil administration

	<u>Diet</u>	<u>Proline transport rate</u> ($\mu\text{M}/100 \text{ cm/h}$)
Control (uninjected)	Diet B	18.0 ± 2.2 (4)
5-fluorouracil (Day 3)	Diet B	3.5 ± 1.3 (6)
5-fluorouracil (Day 3)	Oxoid Diet 86	3.9 ± 1.7 (5)

Proline concentration in the perfusate = 1.3 mmol/l

Values are the mean \pm S.E.M. and the number of animals is shown in parentheses.

for 3 days after 5-fluorouracil administration. For comparison, proline absorption rates were estimated in intestines from rats fed for 10 days on Diet B. The perfusate contained proline (1.3mmol/l); proline was estimated in the serosal secretion collected over a 15-minute period commencing 45 minutes after the start of perfusion. Proline absorption rates were decreased to about 20% of control values ($P < 0.001$) by the third day after 5-fluorouracil administration; the extent was the same regardless of whether the diet was the standard one (Oxoid) or the 'elemental' one (Diet B, Table 10.11). Hence these results are completely at variance with those of Mohiuddin and Kramer (1978) and lead to the same conclusion as those already described above; namely that no beneficial effect of an 'elemental' diet on 5-fluorouracil toxicity has been demonstrated.

DISCUSSION

The results presented in this chapter represent an extensive study of the effects of 'elemental' diets on the intestinal toxicity of 5-fluorouracil in an attempt to confirm and expand upon the observations of Bounous *et al* (1971a and b). In spite of close adherence to the conditions used by Bounous *et al* (1971a) it is concluded that the intestinal toxicity of 5-fluorouracil is not reduced in rats fed on an 'elemental' diet. Previously, Gardner and Heading (1979) came to a similar conclusion. However, as discussed in the introduction to this chapter, there were several inconsistencies between the work of Bounous *et al* (1971a) and that of Gardner and Heading (1979) and these have now been eliminated.

I shall first discuss the effects of 'elemental' diets on the 'normal physiology' of the intestine, and then consider their effects on the pathophysiology seen after 5-fluorouracil administration.

Effects of 'elemental' diets on food intakes and growth rates

Although it is true that rats gained weight when fed on 'elemental' diets, the daily weight gain was 7 to 38% less than that of rats fed on a standard diet (Oxoid). Also, the food intake was less but, because of the high calorific value of these diets (see Tables 10.6 and 10.7), the total daily energy intake was greater, by 11 to 56%, than that of rats fed on the Oxoid diet. Hence the weight gain per kJoule intake was decreased by almost 50% when rats were fed on an 'elemental' diet, suggesting that the energy utilization from 'elemental' diets is very inefficient. These

observations on the growth rates of rats fed on 'elemental' diets contrast with those of Bounous *et al* (1971a), who showed that growth rates were similar in rats fed on hydrolysed and unhydrolysed diets, but agree with those of Gardner and Heading (1979) and those of Bounous and Maestracci (1976), who reported that during the first 10 days the growth rates of rats fed on an 'elemental' diet were 20% less than those of rats fed on an unhydrolysed diet. It is of note that Bounous and Maestracci (1976) do not offer any explanation for the inconsistency of their results and those of Bounous *et al* (1971a). Since it has been shown that the intestinal toxicity of 5-fluorouracil is increased by an overnight fast prior to injection (Chapter 11, Table 11.2), these observations of reduced food intakes and growth rates do not argue in favour of the use of 'elemental' diets during 5-fluorouracil administration.

The results also emphasise the observation, noted in Chapter 8, that the food intakes and growth rates of male rats are greater than those of female rats and indicate a potential advantage of the use of male rats in dietary experiments since the changes in growth rates and food intakes are larger and more easily detected.

Effects of 'elemental' diets on intestinal absorption

Intestinal water absorption rates were increased in rats fed on an 'elemental' diet, although the increase was not always significant (Table 10.9). Gardner and Heading (1979) suggested that the increase might be due to the high carbohydrate content of 'Vivonex' and the results presented in this chapter are consistent with their suggestion. Intestinal water absorption rates in rats fed on 'Vivonex-HN' for 6 weeks were very similar to those observed in rats

fed for 10 days on this diet by Gardner and Heading (1979) (see Table 10.9). The carbohydrate contents of 'Flexical' (68%) and Diet B (54%) are less than that of 'Vivonex-HN' (80%) and these two diets did not produce a significant increase in water absorption rates when fed to rats for 1 week. A significant increase, by 32 and 42%, was observed when 'Flexical' was fed to rats for 2 or 6 weeks respectively (Table 10.9). These results do not agree with recent observations by Nelson *et al* (1980), who reported that both glucose and water absorption rates, estimated *in vivo* in jejunum and ileum, were unchanged in rats fed on 'Vivonex' for 1 month, and were decreased in rats fed on 'Flexical' for 1 month when compared with control animals (diet unspecified). Gardner *et al* (1978) reported a consistent and highly significant positive correlation between glucose and water absorption rates and Gardner and Heading (1979) also observed a similar increase in both glucose and water absorption rates in rats fed on 'Vivonex-HN', suggesting that the correlation is maintained in rats fed on 'elemental' diets. Thus, since water absorption rates were increased in rats fed on 'Flexical' for 2 or 6 weeks, it is probable that glucose absorption rates would also be increased and the difference between the results of Nelson *et al* (1980) and those of Gardner and Heading, the latter confirmed in this study, cannot be explained.

The dry weights of intestines from rats fed on 'Flexical' or on Diet B were significantly greater ($P < 0.001$ for females, $P < 0.02$ for males) than those from rats fed on the standard Oxoid diet (Table 10.9). However, the mucosal DNA content (Fig. 10.4) and cytoplasmic peptide hydrolase activities (Fig. 10.1) were not affected by the 'elemental' diets. This suggests that the increase

in intestinal dry weights and water absorption rates was not due to an increase in the number of cells in the intestinal epithelium. The increase in intestinal dry weight may have been due to accumulation of fat in the intestine which was apparent both by the whitish colour of the intestines and by the high content of fat in the serosal secretion. An increase in the water absorption rate in the absence of a change in the mucosal DNA content suggests that the absorption rate per absorbing cell may have increased. However, as discussed on p 179, this cannot be firmly concluded since the proportions of 'mature' cells may have increased and there is evidence which supports this possibility. Lehnert (1979) showed that when mice were fed on an 'elemental' diet (3200-AS - 'Flexical') instead of on an unhydrolysed diet, the number of cells per villus in the jejunum was increased by 20% and the number of cells per crypt in the jejunum was decreased by 30%.

'Elemental' diets and 5-fluorouracil toxicity

As discussed in Chapter 8, the impairment of intestinal absorption of both glucose and water in the animal model used here is maximal by the third day after 5-fluorouracil administration. The decrease in intestinal dry weight is also maximal on the third day. As can be seen clearly in Fig. 10.6, none of the 'elemental' diets produced a significant increase in intestinal water absorption rates or in intestinal dry weights on the third day after 5-fluorouracil (Tris or sodium salt) administration. Furthermore, the body weight losses over the first 3 days after 5-fluorouracil injection were significantly greater in rats fed on 'Flexical' (31%, $P < 0.01$) or 'Vivonex-HN' (38%, $P < 0.02$) than those of rats

fed on the Oxoid diet (Fig. 10.2). In contrast, Bounous *et al* (1971a) demonstrated a small reduction in body weight losses (about 10%) following 5-fluorouracil administration in rats fed on an 'elemental' diet. However, Stanford *et al* (1977) observed similar weight losses in rats fed on 'Flexical', 'Vivonex' or a standard diet during 5-fluorouracil administration.

A high incidence of diarrhoea following 5-fluorouracil administration was observed in rats fed on the Oxoid diet, 'Flexical' or 'Vivonex-HN'. In contrast, a low incidence was observed in rats fed on Diet B (Table 10.10). Stanford *et al* (1977) also reported a high incidence of diarrhoea (90 - 100%) following 5-fluorouracil administration in rats fed on 'Flexical' or 'Vivonex' but reported a much lower incidence (25%) in rats fed on a standard, unhydrolysed, diet (Purina Rat Chow). Diarrhoea following 5-fluorouracil administration might be due to colonic damage rather than to injury to the small intestine. Thus the lower incidence of diarrhoea following 5-fluorouracil administration in rats fed on Diet B might be due to an alteration of the colonic damage.

The results presented in Figs. 10.2, 10.3 and 10.6 reiterate the observation, discussed in Chapter 8, that the intestinal toxicities of the Tris and sodium salts of 5-fluorouracil are similar, although the effects on body weight and food intakes following administration of the sodium salt of 5-fluorouracil are much less severe than those of the Tris salt. These observations argue against the suggestion of Bounous *et al* (1971a) that the beneficial effects of 'elemental' diets which they observed were due to a direct supply of readily absorbable nutrients to the injured

intestine which aids recovery of the intestine. During the first 48 hours after administration of the Tris salt of 5-fluorouracil the food intake of rats was negligible for all diets and during the next 24 hours the food intake was less than 25% of the normal daily intake (see Chapter 8, Fig. 8.1). In contrast, during the first 48 hours after administration of the sodium salt of 5-fluorouracil, the food intake was maintained at about 50% of the normal intake and during this period there remains a significant digestive and absorptive activity; absorption rates have decreased by only 20% of control rates on the second day after 5-fluorouracil administration (Chapter 8, Fig. 8.3). In spite of this difference in food intakes after administration of the two salts of 5-fluorouracil, the intestinal absorptive activity has decreased to a similar extent. If Bounous et al (1971a) were correct in their suggestion, one would expect that rats injected with the sodium salt of 5-fluorouracil would demonstrate a greater response to 'elemental' diets than would rats injected with the Tris salt of 5-fluorouracil, but this is not so.

When male rats were fed on Diet B a positive correlation was observed between the water absorption rates 3 days after 5-fluorouracil administration and the body weight either at the time of injection or on the third day after injection (Fig. 10.8a and b). Since no correlation was observed with the body weight at the start of dietary modification (Fig. 10.8c) this result suggested that the weight gain over the period that the rats were fed on Diet B might be a relevant factor in the protection of the intestine from 5-fluorouracil toxicity by an 'elemental' diet. However, this theory was not supported by the results obtained from rats caged individually and fed on Diet B with Tomato flavour added. Although

the combination of dietary flavour and individual caging increased the growth rate of rats fed on Diet B to 7% greater than that observed in rats fed on the Oxoid diet (Table 10.6), water absorption rates on the third day after 5-fluorouracil administration were not increased (Fig. 10.6). It is unlikely that the higher absorption rates observed in heavier rats were due to differences in the dosages of 5-fluorouracil since it is given on a body weight basis calculated for each rat. Also, the body weight gains during dietary modification was only about 15% and the intestinal toxicity of 5-fluorouracil has been shown to be equally severe when the dose was reduced from 1.44 mmol/kg to 1.08 mmol/kg (Chapter 8, Fig. 8.8). No explanation can be suggested for the observed correlation and it was not observed subsequently with Diet B or with any of the other diets used.

Effects of 'elemental' diets on the intestinal transport of proline

Mohiuddin and Kramer (1978) reported that proline transport was severely impaired following irradiation of the intestine and that recovery from the injury was much more rapid in rats fed on an 'elemental' diet than in rats fed on an unhydrolysed diet. This observation was consistent with the suggestion of Bounous *et al* (1971a) that 'elemental' diets aid recovery from injury by supplying predigested nutrients directly to the intestine, thereby stimulating a more rapid recovery of absorptive activity. However, in this study, proline transport rates by the small intestine estimated on the third day after 5-fluorouracil administration were similar in rats fed on the Oxoid diet and in rats fed on Diet B, and both were much less (by about 80%) than the rates observed in uninjected rats

(Table 10.11) - a result that is entirely consistent with the conclusion, drawn from the results on water absorption rates, that the 'elemental' diets do not decrease the intestinal toxicity of 5-fluorouracil. The study by Mohiuddin and Kramer (1978) can be criticised on a number of points. Firstly, they used an everted sac preparation incubated in Krebs bicarbonate solution apparently in the absence of glucose. As Fisher (1955) demonstrated, water absorption rates by the perfused small intestine are very low in the absence of glucose in the luminal perfusate since there is no energy source. Also, Mohiuddin and Kramer (1978) express proline absorption rates as the difference in concentration of proline in the sac fluid before and after incubation. Since water absorption rates are severely impaired after 5-fluorouracil administration (Chapter 8, Fig. 8.3, Fig. 10.6, also Gardner et al, 1978) and since the changes in absorptive activity of the small intestine are similar after 5-fluorouracil administration and after irradiation (Robinson 1972), estimations of concentration differences are meaningless unless an estimation of the water absorption rate is also obtained. Furthermore, the relative absorption rates of proline in Mohiuddin and Kramer's (1978) control preparations were 50% greater than the corresponding control values reported in a previous paper (Mohiuddin et al, 1978). Hence, one must question the adequacy of the controls and the consistency of the observations reported by Mohiuddin and Kramer (1978). In view of these criticisms, it must be concluded that there is no evidence to suggest that 'elemental' diets influence beneficially the effects of 5-fluorouracil on intestinal proline absorption.

Effects of 'elemental' diets and 5-fluorouracil on peptide hydrolase activities

Intestinal cytoplasmic peptide hydrolase activities were unaffected 24 hours after 5-fluorouracil administration in rats fed on the Oxoid diet or on 'Flexical' and were decreased to 30 - 60% of normal by the third day (Fig. 10.5). This observation contrasts with that of Bounous et al (1971a) who reported a marked decrease in dipeptide hydrolase activities, to 6 - 12% of normal, both 1 and 3 days after 5-fluorouracil administration. Also, whereas dipeptidase activities were unchanged in intestines from uninjected rats fed on 'Flexical' (Fig. 10.1), Bounous et al (1971a) reported a marked decrease in dipeptidase activities in intestines from rats fed on an 'elemental' diet (Diet B). However, as Gardner and Heading (1979) demonstrated, the effects of 'elemental' diets on intestinal dipeptidase activities depends on the 'elemental' diet and on the particular enzymic activity studied. Since activities against different substrates were estimated, this might explain part of the disagreement between the results of this study and those of Bounous et al (1971a). Furthermore, it is of note that Hugon and Bounous (1972) suggested that the decreased peptide hydrolase activity in intestines from rats fed on an 'elemental' diet which they claimed previously (Bounous et al, 1971a) may have been caused by "a selective decay of the enzyme when the tissue is preserved frozen". This may well have been the cause since it was found that all three activities estimated in this study were decreased upon storage at - 26°C (see p. 45). Also, Bounous et al (1971a) expressed enzyme activity per milligram nitrogen and therefore activities will be affected both by changes in the total activity of the enzyme and by

changes in the composition of the cell cytoplasm. However; Gardner and Heading (1979) also observed similar changes to those reported here when they expressed dipeptidase activity with reference to protein; hence the use of different reference systems may not have contributed to the discrepancy. In view of the effects of 5-fluorouracil on the mucosal cell population (discussed in Chapter 8, p 179); the expression of activity with reference to the whole small intestine, as used in this study, is probably the most useful index of enzyme activity.

It is also of note that Bounous et al (1971a) reported an increase in intestinal sucrase activity 24 hours after administration of 5-fluorouracil. However, sucrase is exclusively a brush border enzyme (Semenza, 1968) and Bounous et al (1971a) estimated this activity in the supernatant fraction of a mucosal homogenate. Since Gardner et al (1978) reported that less than 3% of the intestinal sucrase activity is present in the supernatant, it is not relevant to estimate the activity in this fraction. Hence the activities reported by Bounous et al (1971a) were probably due to a brush border contamination of their supernatant.

Although Bounous et al (1971a) reported that an 'elemental' diet reduced the intestinal toxicity of 5-fluorouracil at a dosage of 150 mg/rat, they did not observe any reduction when the dose was raised to 200 mg/rat. At the higher dose the mortality was similar (64%) in rats fed on an 'elemental' diet and in rats fed on a standard diet. Furthermore, Bounous et al (1978a and b) have since reported that mortality is increased at high doses of 5-fluorouracil (450 mg/kg body weight or 50 mg/kg body weight for

4 days) when rats have been fed on an 'elemental' diet, and these observations have been confirmed by Stanford et al (1977) at a dose of 25 mg/kg body weight for 6 days. However, all of these doses are much greater than the recommended initial clinical dosage in humans of 12 mg/kg body weight for 4 days (Martindale, 1977). Although the dose of 5-fluorouracil used in this study (187 mg/kg) was also greater than the normal human dose, it was less than half the dose at which Bounous et al (1971a) observed the beneficial effects of an 'elemental' diet. Also after injecting more than 500 rats with the sodium salt of 5-fluorouracil at this dose no deaths have occurred. It is presumed that Bounous et al (1971a) used the sodium salt of 5-fluorouracil since Hugon and Bounous (1972) did. Thus, it seems unlikely that the high dose of 5-fluorouracil used in this study has prevented observation of the effects of 'elemental' diets on 5-fluorouracil toxicity reported by Bounous et al (1971a). Furthermore, if the mortality is increased in rats fed on an 'elemental' diet then this must argue against the use of 'elemental' diets, regardless of their effects on intestinal function.

It must therefore be concluded that the intestinal toxicity of 5-fluorouracil is not significantly reduced in rats fed on an 'elemental' diet. Bounous et al (1971a) claimed that 'elemental' diets aid recovery from injury since the degree of mitotic arrest in the intestine was similar for rats fed on an 'elemental' diet and for rats fed on a standard diet. The mitotic arrest results in a 65% reduction in mucosal DNA content (Fig. 10.4) and hence a large reduction in the number of epithelial cells. The malabsorption symptoms following 5-fluorouracil administration are probably a direct result of this decrease in the epithelial cell population

(see Chapter 8). It would, therefore, appear more profitable to prevent this decrease in cell numbers rather than to aid recovery which is already a rapid process.

CHAPTER 11

The effects of the time of feeding and the time of 5-fluorouracil administration on the intestinal toxicity of 5-fluorouracil -

A 'Chronopharmacological' approach

Introduction

The severe impairment of intestinal absorption observed following administration of 5-fluorouracil is probably a direct result of a reduction in the number of intestinal absorptive cells (Chapter 8). In Chapter 10 it was concluded that 'elemental' diets do not protect either the whole body or the small intestine from the toxic effects of 5-fluorouracil and that attempts to reduce this toxicity should instead be aimed at reducing the initial damage to intestinal cells. Ecknauer and Rommel (1978) suggested, in principle, that this might be achieved by relating the time of administration of an antimetabolite to the cell cycle of the mucosal cells. One might expect that least damage to the cells would result if the drug were administered during the resting phase (G_1) of the cell cycle.

Skipper *et al* (1967) reported that the ability of cytosine arabinoside to kill mouse leukaemia cells is greatly dependent upon the interval between administration of sequential doses of the drug and that the drug is most effective when the interval takes advantage of the S-phase specificity of the antimetabolite. Also, Devik and Hagen (1973) found that the general systemic toxicity of various anti-cancer agents, estimated by body weight loss and mortality, depended on the time interval between administration of sequential doses of the drugs: for hydroxyurea and methotrexate they suggested that this

effect was related to the timing of the cell cycle. Haus et al (1974) presented results to show that the timing of irradiation can influence the severity of whole body and gastrointestinal radiation damage. Willems (1979) in his authoritative review in The Scientific Basis of Gastroenterology stated that this was the case, but did not give any supporting evidence or references.

This general approach has been advocated by others especially in the U.S.A. where the term 'chronopharmacology' has been coined (see Reinberg and Halberg, 1979) but has received little attention in this country. Although an Editorial in the British Medical Journal (1978) reviewed favourably the application of 'chronotherapeutics', this aspect of therapeutics is not widely recognised, and certainly is seldom exploited. However, observations mentioned above indicate that substantial benefits in terms of reduced toxicity of antimetabolites might be achieved in this way.

Many antimetabolites including hydroxyurea and cytosine arabinoside are only toxic to cells actually synthesising DNA (i.e. cells in S-phase) (Heidelberger, 1973; Lozzio, 1969; Sinclair, 1965). However, it has been suggested that cells are susceptible to 5-fluorouracil and the related compound fluorodeoxyuridine in all phases of the cell cycle (Bruce and Meeker, 1967; Lozzio, 1969) although 5-fluorouracil is more toxic to rapidly proliferating cells than to non-proliferating cells (Bruce and Meeker, 1967). Therefore 5-fluorouracil administration time cannot be related to the cell cycle in order to reduce the intestinal toxicity of the drug but it might be possible to relate the drug administration time to the diurnal variation in intestinal cell proliferation and in the number of villus cells (Al-Dewachi et al, 1976; Stevenson et al, 1979). These diurnal variations as well as

those of various intestinal absorptive and enzymic activities have been shown to be related to the feeding time in rats (Fisher and Gardner, 1976; Stevenson et al, 1975; Stevenson and Fierstein, 1976; Stevenson et al, 1979).

All these observations point to the potential value of exploring the temporal relationships between 5-fluorouracil injection time, the cell proliferation cycle, animal feeding time and the subsequent 5-fluorouracil toxicity; this chapter is therefore devoted to such an approach to minimizing 5-fluorouracil toxicity on the rat small intestine. Since the intestinal toxicity of 5-fluorouracil might depend upon the proliferation cycle in the intestinal epithelium, which in turn is influenced by the feeding time of the animals, there are two possible approaches to such a study. Both of these have been investigated - by systematic experimental variation in feeding time while keeping 5-fluorouracil administration time constant and by systematic variation in 5-fluorouracil administration time while keeping feeding time constant.

Initial observations indicated that 5-fluorouracil was more toxic to the intestine when administered in the afternoon than when administered in the morning and that the toxicity was increased by fasting the rats overnight prior to 5-fluorouracil injection. These differences in toxicity might, however, be related to the effects of feeding time on the proliferative cycle in the intestinal epithelium. Further, since rats are nocturnal feeders, these differences could indicate a direct relationship between either the presence of food in the intestine or the digestive and absorptive activity of the intestine at the time of injection and the subsequent toxicity of 5-fluorouracil. Both of these possibilities have been investigated in a study of the

effects of manipulating the feeding time of rats on the intestinal toxicity of 5-fluorouracil. As will be discussed, this approach had various shortcomings and was eventually discontinued in favour of the alternative approach in which the effects of a systematic variation in injection time on the intestinal toxicity of 5-fluorouracil were studied concurrently with observations of the feeding pattern on various diets. This chapter describes the results obtained in these two series of experiments.

METHODS

Preliminary studies of the effects of injection time

Initially, 5-fluorouracil (Tris salt, 1.44 mmol/kg body weight) was administered intraperitoneally to female rats at either 09:00 or 15:30 and water absorption rates by the whole jejunum plus ileum were estimated in vitro on the fifth day after 5-fluorouracil administration. Although maximal intestinal toxicity is observed on the third and fourth days after injection of 5-fluorouracil (Chapter 8), absorption rates were estimated on the fifth day in order that the $6\frac{1}{2}$ hour difference in injection time would be small relative to the interval between injection and absorption measurement. The rats had free access to the Oxoid diet for at least 7 days prior to 5-fluorouracil administration and thereafter.

In a second experiment, the effects of intestinal digestive activity, i.e. the presence of food in the gut at the time of 5-fluorouracil administration, were investigated. 5-fluorouracil (Tris salt, 1.44 mmol/kg body weight) was administered near to the end of the normal feeding period (07:00) to two groups of female rats; one group had free access to Oxoid for 7 days prior to 5-fluorouracil administration and the other group was fasted from 11:00 on the day prior to 5-fluorouracil administration. All rats had free access to the Oxoid diet after injection. Water absorption rates by the whole jejunum plus ileum were estimated in vitro on the third day after drug administration.

Restriction of feeding time

5-fluorouracil (Tris salt, 1.44 mmol/kg body weight) was administered intraperitoneally to rats at a fixed time, viz. 10:00;

rats were from two groups, one of which was fed immediately before the time of drug administration and the other was fed 12 hours prior to the time of drug administration. Since rats fed ad lib consume 40% of their whole days food intake between the hours of 19:00 and 22:00 (see Fig. 11.14b), and since preliminary observations indicated that rats whose feeding was restricted to 3 hours each day would adapt to consume the same amount of food as that consumed by rats fed ad lib, the two groups were fed for only 3 hours each day, either between 07:00 and 10:00 or between 19:00 and 22:00, for two weeks prior to 5-fluorouracil administration. A short feeding period has the advantage that it minimises variability in feeding times between animals. Also, Fisher and Gardner (1976) had claimed that normal growth rates were observed in rats fed for only 3 hours each day.

In most experiments access to food was limited by use of the automatic feeder cages described in Chapter 2 (p. 15). However, this method was not suitable for use with a non-pelleted 'elemental' diet and therefore when this diet, Diet B, was used and in some experiments where the Oxoid diet was used, access to food was limited manually.

In all experiments in which access to food was limited, the rats were caged in groups of 4 since this was the maximum number of rats that could be housed together such that all rats showed an overall weight gain over the 14 day period of restricted access to food (see p 286). It was not practical to put less than 4 rats in each cage due to lack of space in the animal house although this would have been ideal.

Body weights and food intakes were recorded daily and the feeding regimes were continued after 5-fluorouracil administration. Control

rats received no injection.

Water absorption rates by the whole jejunum plus ileum were estimated in vitro by the method described in Chapter 3 and all measurements were made between 10:00 and 16:00 on the third day after 5-fluorouracil injection or, in control rats, on the 17th day of the food restriction regime (i.e. 14 days + 3 days).

Variation of 5-fluorouracil administration time

5-Fluorouracil (sodium salt, 1.44 mmol/kg body weight) was administered intraperitoneally to male rats at various times of the day and night. All injections were given within 10 minutes of the specified time. Body weights, food intakes and the incidence of diarrhoea were recorded subsequently at 10:00 each day. The last was obvious upon inspection of the rats.

Initially, all the rats had free access to the Oxoid diet before and after 5-fluorouracil administration. In some instances, specified below, the diet was replaced by an 'elemental' diet (Diet B) and in some other experiments access to the diet (Oxoid or Diet B) was restricted to the period 19:00 to 22:00 for 14 days prior to 5-fluorouracil administration. All rats were caged in groups of 4 unless otherwise stated and had free access to food after 5-fluorouracil administration.

Absorption rates were measured between 3 and 4 days after 5-fluorouracil injection since the lowest absorption rates following injection at 10:00 had been observed on the third and fourth days (see Chapter 8, Fig. 8.3). Since there is a diurnal variation in water absorption rates, all absorption measurements were made between 10:00

and 16:00. Thus, absorption rates were estimated on the third day after injection between 00:00 and 11:00 inclusive and on the fourth day after injection between 15:00 and 23:00 inclusive.

The dry weight of intestines was not used as a parameter to estimate intestinal damage in these experiments. Because of the diurnal variation in absorption rates it is not possible to estimate absorption rates and dry weight at a fixed time after administration of 5-fluorouracil. Absorption rates were similar on the third and fourth days after injection of the drug at 10:00 and therefore it is probable that any variation in the absorption rates estimated between the third and fourth days will be due to the variation in drug administration time. However, there is an increase in the intestinal dry weight between the third and fourth days after administration of 5-fluorouracil at 10:00 (Chapter 8, Fig.8.4) and therefore variations in the intestinal dry weight between the third and fourth days after drug administration cannot solely be attributed to the time of drug administration.

Plasma pharmacokinetics

Male rats, weighing 200 g, were anaesthetised 1 hour before use by subcutaneous injection of urethane (1.5 ml of 0.8 g/ml solution) while under temporary light ether anaesthesia. Plasma pharmacokinetics were estimated after an intraperitoneal injection of 5-fluorouracil (sodium salt, 1.44 mmol/kg body weight) at either 10:00 or 02:00 by the method described in Chapter 9.

DNA estimation

The DNA content of the mucosa from whole jejunum plus ileum from

control, uninjected, male rats was estimated at various times of the day and night by the method described in Chapter 5.

Feeding pattern

The hour to hour food consumption of rats was determined by changing the lid of the cage which contained the food, or by removal of the food bowl when an 'elemental' diet was used, at 1 hour intervals and noting the weight of food remaining. Special care was taken to minimise the disturbance to the animals; thus, the cage lid was replaced by one into which the weighed diet had already been quietly placed, and the lighting in the animal rooms was not interfered with even though it became necessary to perform these operations in the dark. All rats were allowed 1 week prior to observation to adjust to the housing or dietary regime. They were either caged individually or in groups of 4 and fed on the Oxoid diet or they were caged in groups of 4 and fed on the 'elemental' diet, Diet B. In order to determine whether the hourly disturbance associated with this method of observation materially influenced the feeding pattern of the rats a further two cages of 4 rats, fed on the Oxoid diet, were observed at 2 hour intervals.

Table 11.1 Intestinal water absorption rates and dry weights on the
fifth day after 5-fluorouracil (Tris salt) administration to female
rats at either 09:00 or 15:30

<u>Injection</u> <u>time</u>	<u>Water absorption rate</u> <u>(μl/cm/hr)</u>	<u>% of control</u>
09:00	106.7 \pm 13.5 (7)	76.9
15:30	52.3 \pm 7.8 (7)	37.7
	**	
	<u>Intestinal dry weight</u> <u>(mg/cm)</u>	
09:00	10.72 \pm 0.48 (7)	93.1
15:30	8.34 \pm 0.38 (7)	72.5
	**	

Values are the mean \pm S.E.M. and the number of animals is shown in parentheses. The significance levels of the differences between injection times are indicated thus: ** $P < 0.01$.

RESULTS

Preliminary observations on the effects of injection time on intestinal absorption and dry weight

Table 11.1 shows the water absorption rates and dry weights of intestines from rats which had been injected with 5 -fluorouracil 5 days previously at either 09:00 or 15:30. The results are also expressed as a percentage of those from control (uninjected) rats. On the fifth day after 5-fluorouracil administration at 09:00 intestinal water absorption rates were 77% of control rates, which is significantly greater ($P < 0.01$) by a factor of 2 than those observed on the fifth day after administration of 5-fluorouracil at 15:30. Similarly, the dry weights of intestines from rats injected at 09:00 were significantly greater ($P < 0.01$) by 29% on the fifth day after injection than those from rats injected at 15:30.

Effects of previous starvation on the intestinal toxicity of 5-fluorouracil

Table 11.2 shows the water absorption rates and dry weights of intestines from rats which had been injected 3 days previously with 5-fluorouracil at 07:00. The rats had either had free access to Oxoid prior to 5-fluorouracil injection or had been fasted for 20 hours prior to injection.

Water absorption rates on the third day after 5-fluorouracil administration were decreased by 72% in 'fed' rats and by 88% in fasted* rats and the difference was significant ($P < 0.001$). Similarly the

* Both are compared with 'fed' control rates

Table 11.2 Intestinal water absorption rates and dry weights on the
third day after 5-fluorouracil (Tris salt) administration to female
rats at 07:00 to fed and fasted rats

<u>Injection</u> <u>time</u>	<u>Water absorption rate</u> <u>(μl/cm/hr)</u>	<u>% of fed</u> <u>controls</u>
07:00	$38.5 \pm 2.3 (6)$	27.8
07:00 after 20 hour fast	$16.2 \pm 4.3 (4)$	11.7
	} ***	
	<u>Intestinal dry weight</u> <u>(mg/cm)</u>	
07:00	$6.59 \pm 0.60 (8)$	57.3
07:00 after 20 hour fast	$5.23 \pm 0.28 (6)$	45.4
	} **	

Values are the mean \pm S.E.M. and the number of animals is shown in parentheses. The significance levels of the differences are indicated thus: *** $P < 0.001$; ** $P < 0.02$.

decrease in intestinal dry weight on the third day after 5-fluorouracil injection was significantly greater in fasted rats (55%) than in 'fed' rats (43%, $P < 0.002$).

Effects of restricted access to food on food intakes and growth rates

The results shown in Tables 11.1 and 11.2 show that the intestinal toxicity differs with the time of injection of 5-fluorouracil; this might be related to the feeding time of the rats or to the functional activity of the small intestine at the time of 5-fluorouracil administration. These possibilities have been investigated by determining the effects of two different feeding regimes on the intestinal toxicity of 5-fluorouracil. Animals were fed either immediately before the time of 5-fluorouracil injection or some 12 hours previously.

Fig. 11.1 shows the daily food intake of two groups of rats fed ad lib on the Oxoid diet for five days and then allowed access to the Oxoid diet between 19:00 and 22:00 (Fig. 11.1A) or between 07:00 and 10:00 (Fig. 11.1B). After a decrease in the daily food intake at the start of the period of restricted access to food there was a gradual daily increase in food intake such that by the eighth day of the dietary regime the daily food intake was within the normal range of daily food intakes observed when the rats had free access to Oxoid. A similar pattern was observed when the Oxoid diet was replaced by an 'elemental' diet, Diet B (results not presented).

Although the daily food intake of rats with restricted access to the Oxoid diet became similar to that of rats fed ad lib, the growth rate of the rats with restricted access to food was decreased. Table 11.3 shows the growth rate of rats with free access to food and of those that had access to food for only 3 hours each day. The growth rate of

Fig. 11.1 Daily food intakes of male rats fed ad lib on the Oxoid diet for 5 days and then allowed access to food only between 19:00 and 22:00 (A) or between 07:00 and 10:00 (B) each day for the next 14 days. Results are the mean daily food intake per rat from 1 cage of 4 rats for each dietary regime.

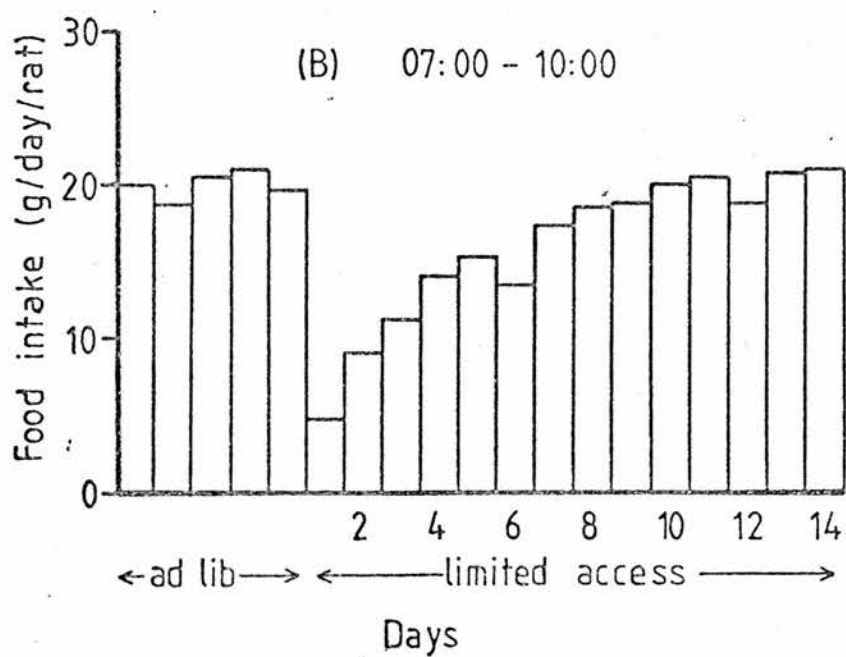
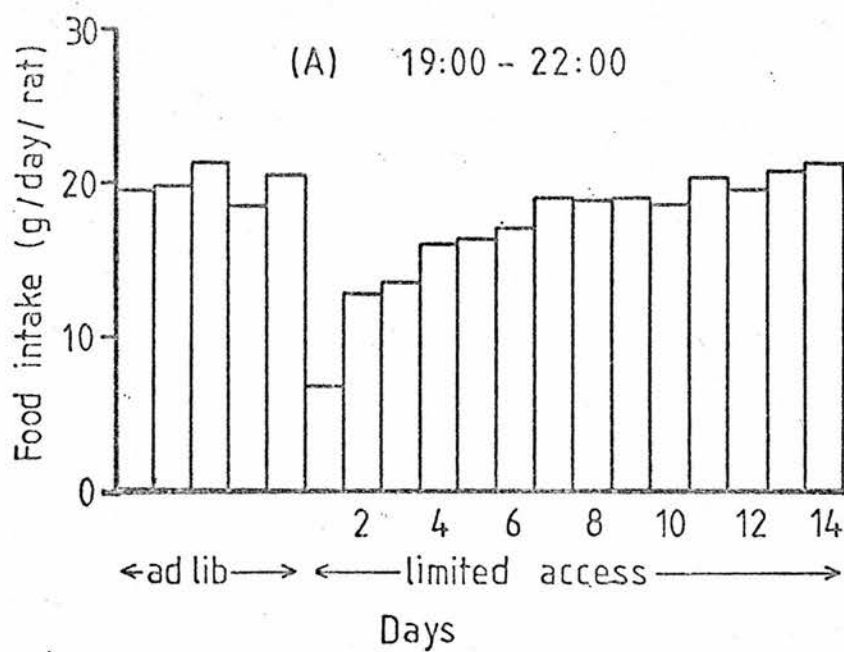


Table 11.3 Daily body weight gains of male rats on various dietary regimes

<u>Diet</u>	<u>Feeding time</u>	<u>Daily body weight gain (g/day/rat)</u>	<u>% of gain on ad lib regime</u>
Oxoid	<u>ad lib</u>	6.05 \pm 0.21 (34)	100
Diet B	<u>ad lib</u>	5.31 \pm 0.35 (32)	100
Oxoid	07:00 - 10:00	5.22 \pm 0.19 (32)	86.3
Diet B	07:00 - 10:00	5.33 \pm 0.40 (34)	100.4
Oxoid	19:00 - 22:00	4.41 \pm 0.34 (40)	72.9
Diet B	19:00 - 22:00	5.43 \pm 0.23 (48)	102.3

Results are the mean \pm S.E.M. and the number of observations is shown in parentheses. When rats had restricted access to food 1 observation refers to the mean body weight gain of a rat between the seventh and fourteenth days of the dietary regime.

rats fed on Oxoid was decreased by 14% ($P < 0.01$) when access was limited to between 07:00 and 10:00 and by 27% ($P < 0.001$) when access was limited to between 19:00 and 22:00.

However, when access to Diet B was limited to only 3 hours each day both the food intake and the growth rate between the eighth and fourteenth days of the dietary regime were equal to those of rats with free access to Diet B. It should be noted, however, that the growth rate of rats with free access to Diet B was significantly less by 12% ($P < 0.01$) than that of rats with free access to the Oxoid diet.

Effects of animal housing on growth rates

Figs. 11.2 and 11.3 show the body weights of individual rats on each of the first 14 days of a food restriction regime. The rats were fed on Oxoid between 19:00 and 22:00 and were caged in a group of 5 (Fig. 11.2) or in a group of 4 (Fig. 11.3). After an initial loss of body weight a steady growth rate was observed when rats were caged in a group of 4 and the mean weight gain over the 14 day period was 34.3 ± 5.8 g/rat. However when rats were housed in a group of 5 the mean weight gain over the 14 day period was only 11.8 ± 6.1 g/rat and one rat (Fig. 11.2D) failed to regain its initial body weight. The food intakes of these two groups of rats also differed. Whilst that for the group of 4 (19.5 ± 0.42 g/day/rat) was not significantly different from the food intake of rats fed ad lib ($P < 0.5$) the food intake of the group of 5 (15.0 ± 0.71 g/day/rat) was significantly less ($P < 0.02$).

Effects of restricted access to food on the intestinal toxicity of 5-fluorouracil

Fig. 11.4 (open bars) shows the water absorption rates by the

Fig. 11.2

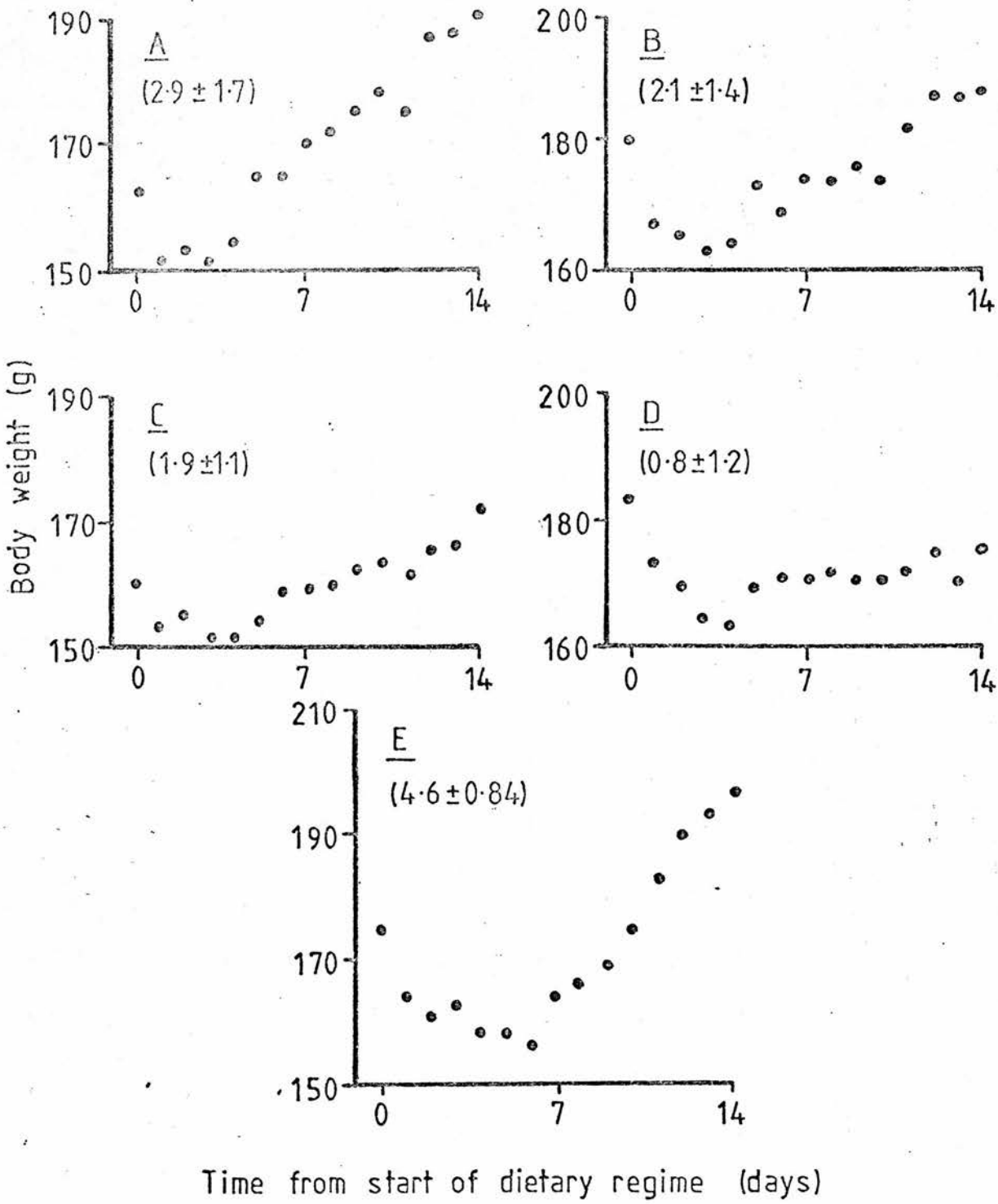
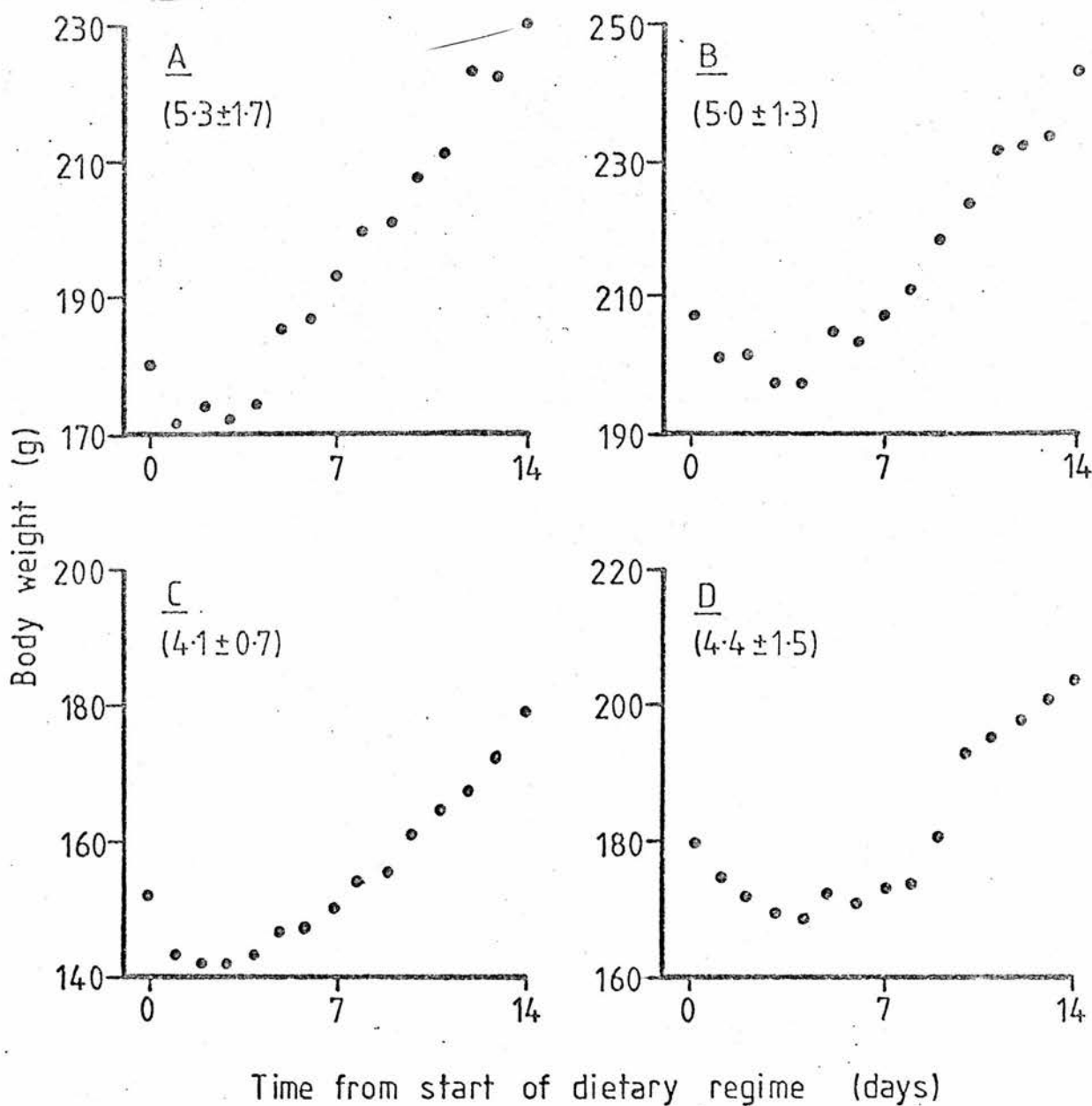
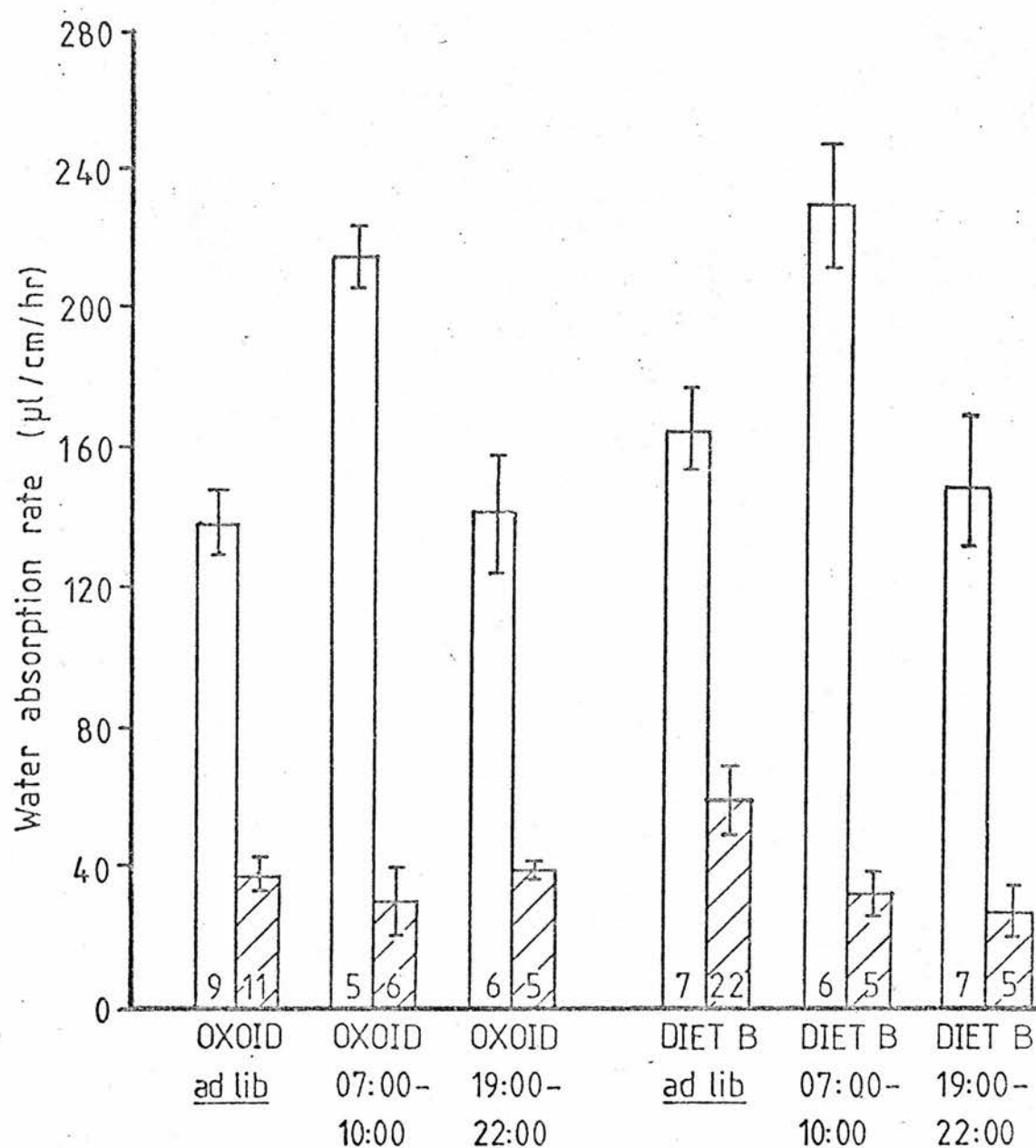


Fig. 11.3



Figs. 11.2 and 11.3 Daily body weights of individual rats on each of the first 14 days of a dietary regime in which rats were only allowed access to food (Oxoid diet) between 19:00 and 22:00 each day. The rats were either caged in a group of 5 (Fig. 11.2) or in a group of 4 (Fig. 11.3). The mean daily body weight gain of each rat between the seventh and fourteenth days of the dietary regime is shown in parentheses.

Fig. 11.4 Intestinal water absorption rates by the small intestine from rats fed ad lib on the Oxoid diet or Diet B and of rats fed on the Oxoid diet or Diet B between 07:00 and 10:00 or between 19:00 and 22:00 each day (open bars). Also shown are the water absorption rates estimated on the third day after 5-fluorouracil injection at 10:00 on the fourteenth day of each of these dietary regimes (hatched bars). Values are the mean \pm S.E.M. and the number of observations is shown within the bars.



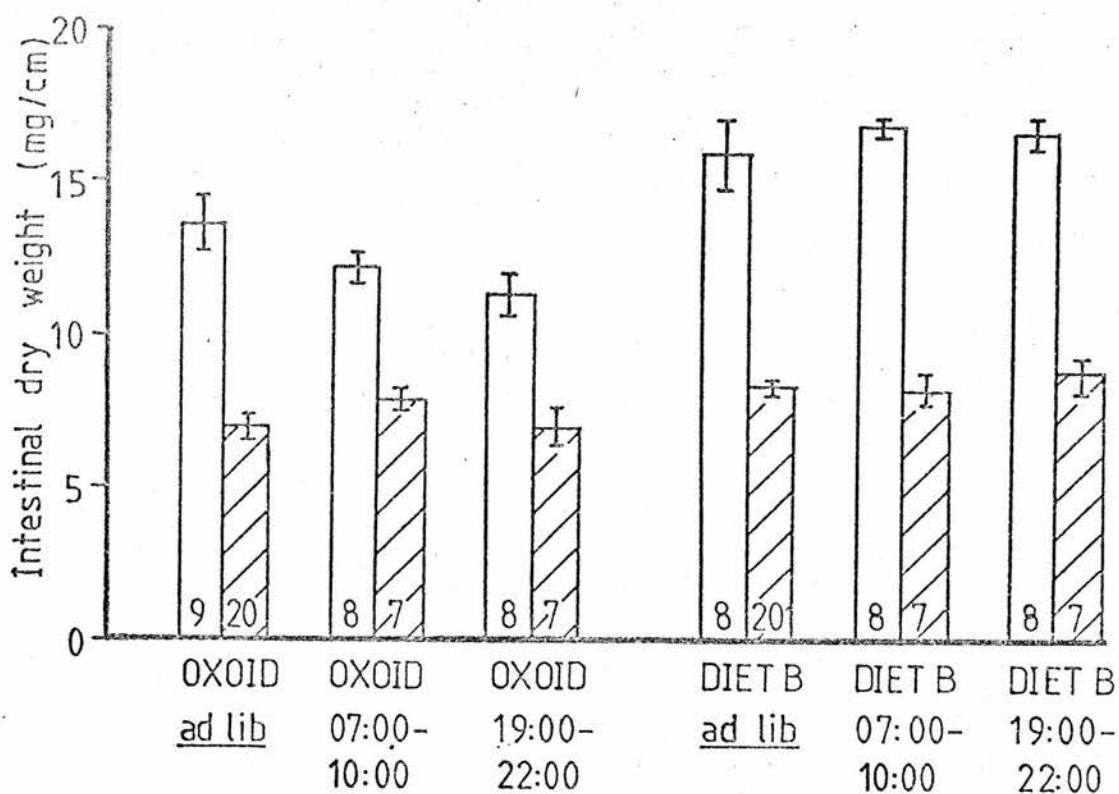


Fig. 11.5 Intestinal dry weights from rats fed on the Oxoid diet or Diet B ad lib or between 07:00 and 10:00 or between 19:00 and 22:00 each day (open bars). Also shown are the intestinal dry weights measured on the third day after 5-fluorouracil injection at 10:00 on the fourteenth day of each of these dietary regimes (hatched bars). Values are the means \pm S.E.M. and the number of observations is shown within the bars.

small intestine of rats fed on the Oxoid diet or on Diet B either ad lib or for only 3 hours each day. Also shown (hatched bars) are the absorption rates on the third day after 5-fluorouracil injection at 10:00 on the fourteenth day of each of these dietary regimes.

When access to the Oxoid diet or to Diet B was restricted to between 07:00 and 10:00 each day, water absorption rates by control intestines (uninjected rats) were significantly higher ($P \leq 0.001$), by 56% and 39% respectively, than those from rats fed ad lib on the same diet. In contrast, water absorption rates were the same when access to food was limited to between 19:00 and 22:00 each day ($P > 0.5$) as when unrestricted access to food was given.

However, neither regime had any effect on the intestinal toxicity of 5-fluorouracil when rats were fed on the Oxoid diet. Although the intestinal water absorption rate on the third day after 5-fluorouracil administration was slightly greater when rats were fed on Diet B ad lib rather than the Oxoid diet ad lib, this difference was not significant ($P > 0.1$).^{*} However, when access to Diet B was limited to only 3 hours in each day the water absorption rates were significantly lower on the third day after 5-fluorouracil administration than those observed when the rats had free access to Diet B prior to 5-fluorouracil injection ($P < 0.01$).

The dry weight of the intestine of control (uninjected) rats was not affected when access to the Oxoid diet was limited to between

* The skewed distribution of absorption rates 3 days after 5-fluorouracil administration to rats fed ad lib on Diet B indicating that this diet may be of benefit to some rats is discussed further in Chapter 10.

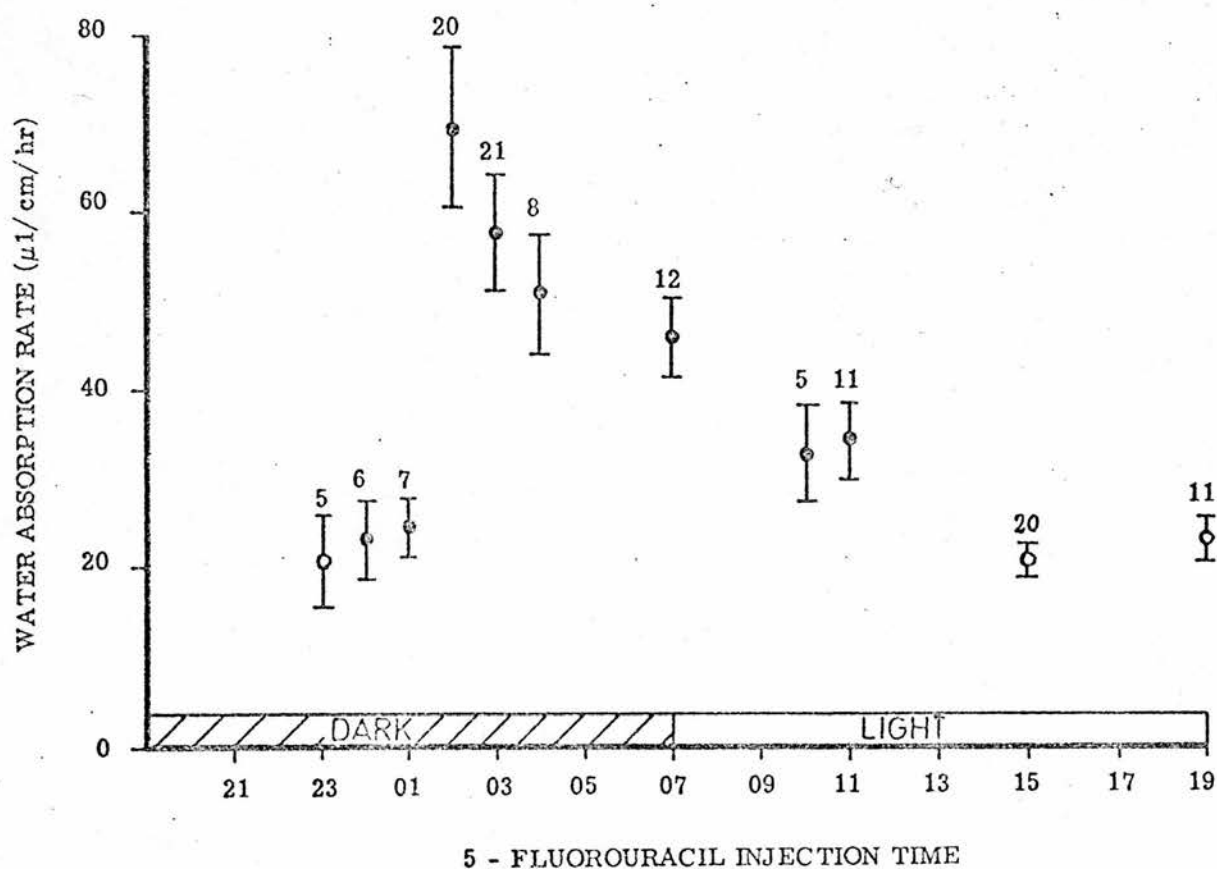


Fig. 11.6 Water absorption rates by rat small intestine 3 or 4 days after 5-fluorouracil injection at various times of the day and night. Absorption rates were measured on the third day after injection between 00:00 and 11:00 inclusive (●) and on the fourth day after injection between 15:00 and 23:00 inclusive (○). Values are the means \pm S.E.M. and the number of animals used is shown above each point. The timing of the light-dark cycle is shown on the abscissa.

07:00 and 10:00 each day but it was decreased by 18% ($P \leq 0.05$) when access to the Oxoid diet was limited to between 19:00 and 22:00 (Fig. 11.5). The intestinal dry weight was significantly greater, by 17%, when Diet B was supplied rather than the Oxoid diet ($P \leq 0.02$), and this was also observed when access to the diet was limited to only 3 hours each day. The intestinal dry weight on the third day after 5-fluorouracil administration was also greater, by about 18% ($P \leq 0.05$) when rats were fed on Diet B rather than on the Oxoid diet.

Effects of injection time on water absorption and the incidence of diarrhoea

Fig. 11.6 shows the intestinal water absorption rates measured in vitro between 3 and 4 days after 5-fluorouracil administration at various times of the day and night. It is clear that the water absorption rates varied systematically with the time of 5-fluorouracil administration. The highest absorption rates were observed following injection at 02:00 and these were greater by a factor of 3 than those observed after injection at 01:00 ($P \leq 0.01$). Absorption rates after injection at all other times were less than those after injection at 03:00. Furthermore, the least incidence of diarrhoea in vivo occurred following injection at 02:00 and 03:00 (Fig. 11.7). Administration of 5-fluorouracil at all other times was associated with a remarkably high incidence of diarrhoea and an obvious general malaise in the rats by the third day after drug administration. This difference is clearly demonstrated in Plate 11.1 which shows the general appearance of two rats on the third day after 5-fluorouracil administration. One rat was injected at 02:00 (Plate 11.1A) and the other at 15:00 (Plate 11.1B).

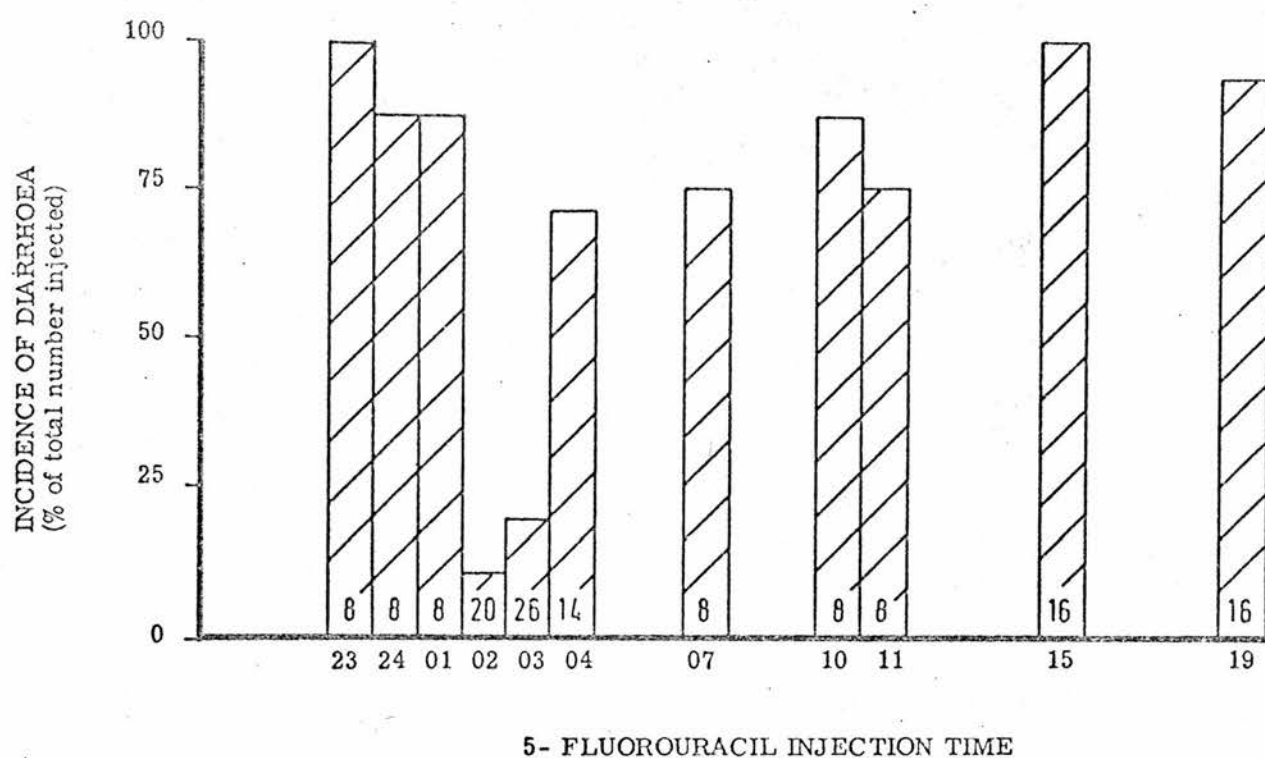


Fig. 11.7 The incidence of diarrhoea observed over 3 days immediately after a single injection of 5-fluorouracil administered at various times of the day and night. Values are the percentage of all animals injected (total number shown within the bars) in which diarrhoea was observed.

Plate 11.1

(A) Injection time 02:00



(B) Injection time 15:00



However, the injection time had no obvious effect on the food intake of these rats during the period between drug administration and estimation of intestinal water absorption (Fig. 11.8). In order to allow for the hour to hour variation in food intake and to compare the food intakes of the rats they were expressed as a percentage of the expected food intake of uninjected rats over the same period which was calculated from the results shown in Fig. 11.14b.

Water absorption rates on each of the first 7 days after 5-fluorouracil injection at 02:00 and 15:00

Two injection times were then selected representative of minimum and maximum intestinal toxicity, 02:00 and 15:00 respectively. Fig. 11.9 shows the water absorption rates measured on each of the first 7 days after 5-fluorouracil injection at these two times. Apart from an initial rise on the first day after administration at 15:00, the absorption rates were consistently higher after injection at 02:00 than after injection at 15:00. Absorption rates have returned to normal between the sixth and seventh days after injection at either time and were slightly, though not significantly ($P > 0.1$) greater than control rates by the seventh day.

The maximum decrease in body weight occurred on the fourth day after injection at 02:00 when the body weight was 88% of that prior to injection (Fig. 11.10). In contrast, after injection at 15:00 the body weight decreased further between the fourth and fifth days, such that by the fifth day it was only 82% of that prior to injection.

The time of injection did not affect the pattern of change in intestinal dry weight (Fig. 11.11) or the food intake (results not

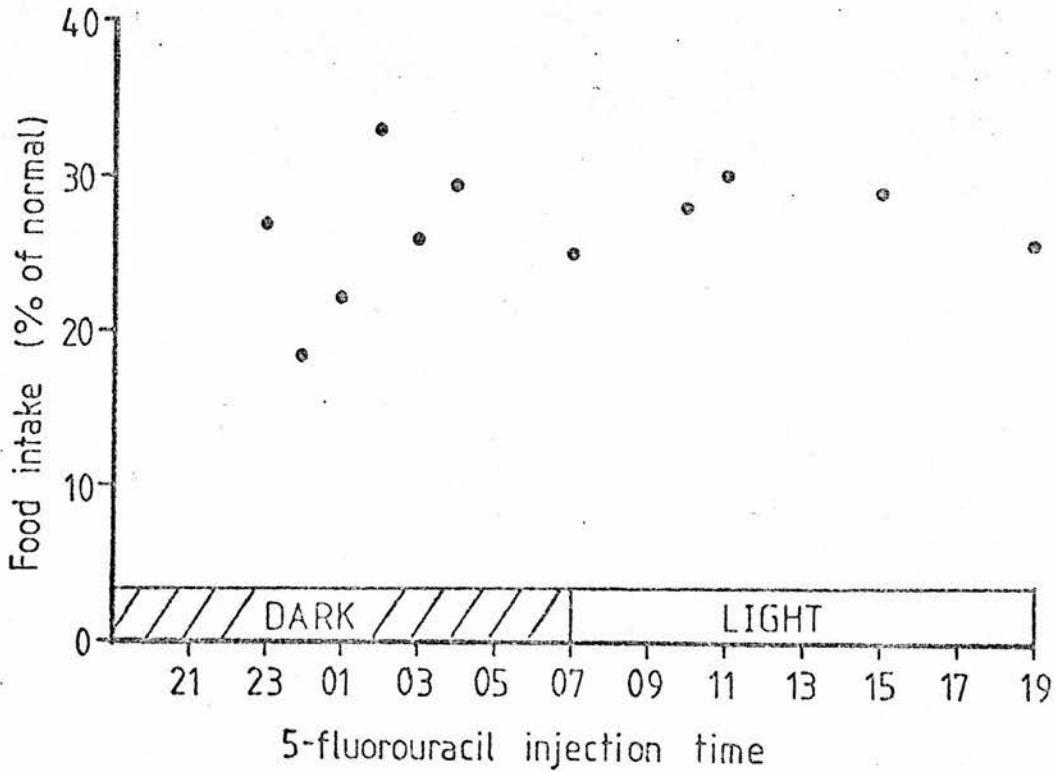


Fig. 11.8 Total food intake over the three to four day period between 5-fluorouracil administration and estimation of intestinal absorption of male rats injected with 5-fluorouracil at various times of the day and night. Values are expressed as a percentage of the normal food intake of uninjected rats over a similar period. Estimates of food intake were obtained from 2 cages of 4 rats for each injection time. The timing of the light-dark cycle is shown on the abscissa.

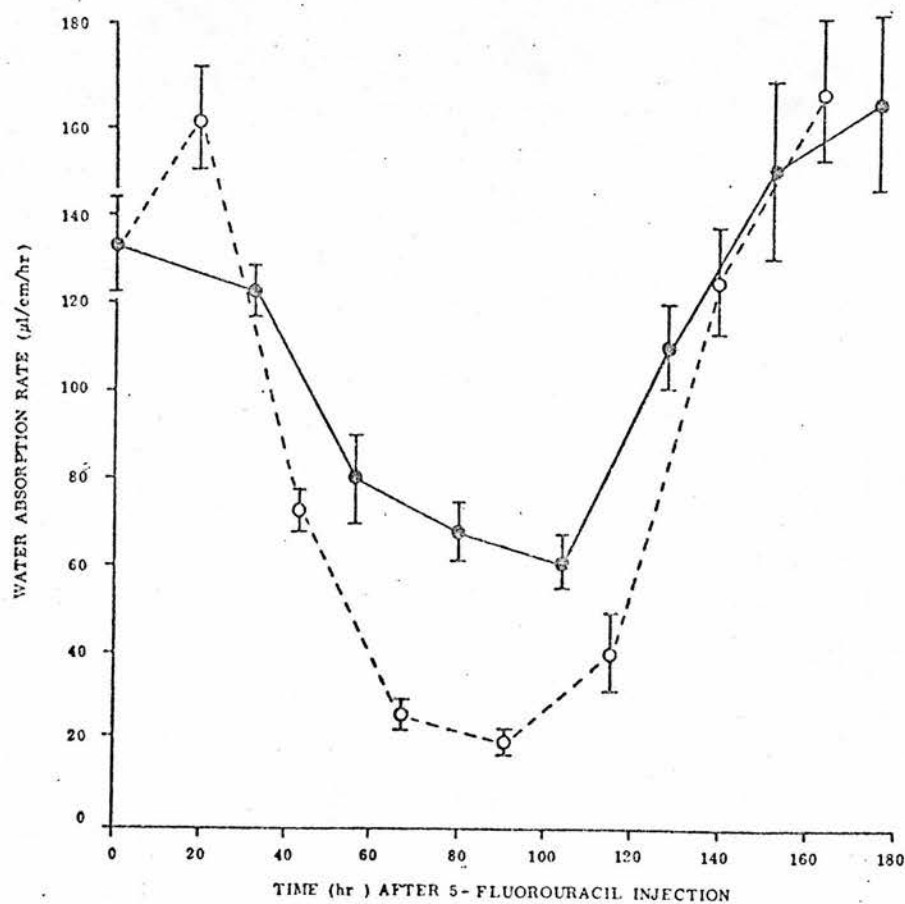


Fig. 11.9 Water absorption rates by rat small intestine on each of the first 7 days following 5-fluorouracil injection at 02:00 (solid line) and 15:00 (broken line). Absorption rates (means \pm S.E.M.) are plotted against the interval between injection and absorption measurement. Each point represents the mean from at least 6 animals.

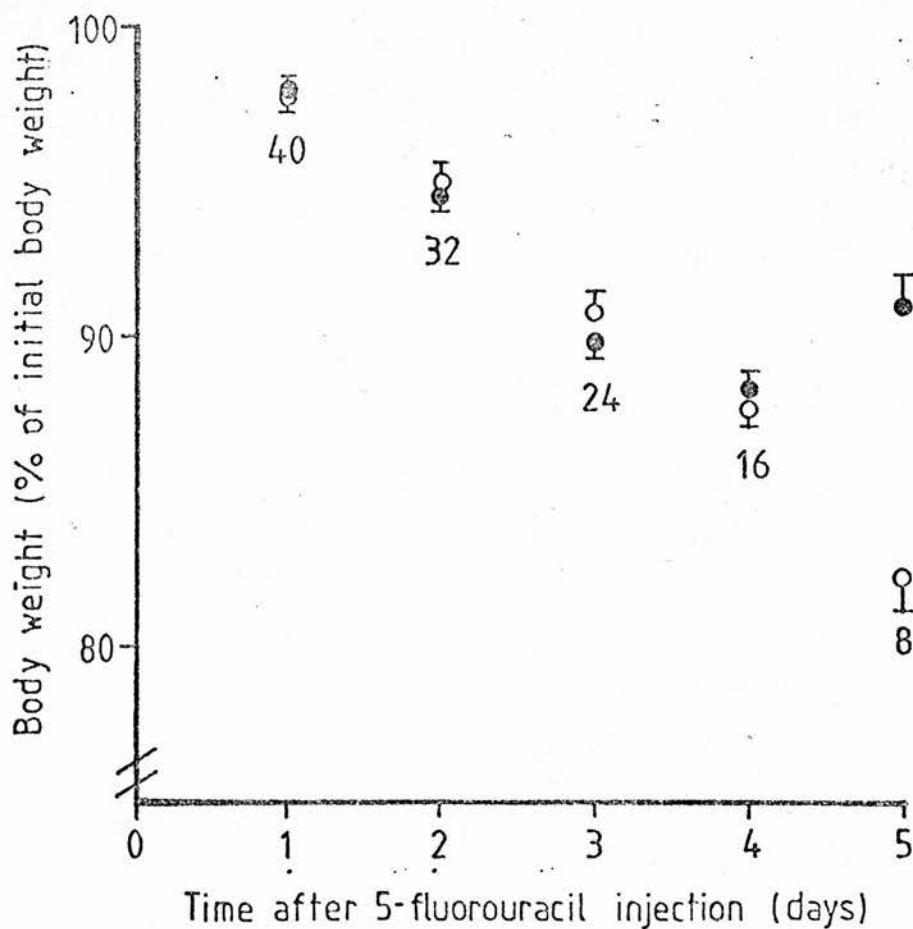


Fig. 11.10 Body weights of rats on each of the first 5 days after 5-fluorouracil administration at either 02:00 (solid circles) or 15:00 (open circles). Body weights (mean \pm S.E.M.) are expressed as a percentage of the body weight at 10:00 on the day before 5-fluorouracil administration. The number of observations for both injection times were the same and are shown below the points.

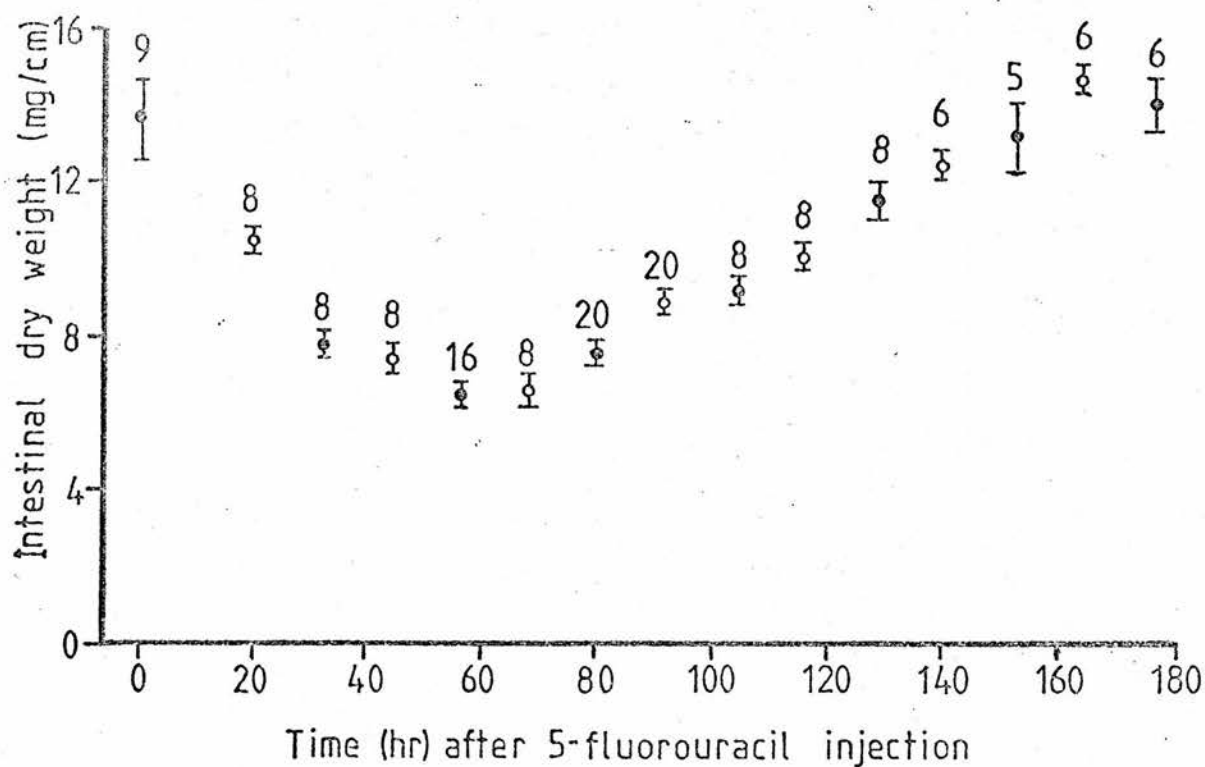


Fig. 11.11 Dry weights of rat small intestine on each of the first 7 days following 5-fluorouracil injection at 02:00 (●) and 15:00 (○). Dry weights (mean \pm S.E.M.) are plotted against the interval between injection and removal of the intestine for dry weight measurement. The number of animals used is shown above each point.

presented but see Fig. 11.8).

Plasma pharmacokinetics of 5-fluorouracil after administration at 02:00

The decreased intestinal toxicity of 5-fluorouracil after administration at 02:00 could be the result of a difference in either absorption or metabolism of the drug at this time. This possibility was investigated by measurement of the plasma pharmacokinetics of 5-fluorouracil after an intraperitoneal injection at either 02:00 or 10:00. There was no significant difference in the plasma levels of 5-fluorouracil during the first 240 minutes after 5-fluorouracil administration at these two times (Fig. 11.12). The number of animals studied, however, is insufficient to permit the definite exclusion of this possibility.

Mucosal DNA content

Fig. 11.13 shows the DNA content of the small intestinal mucosa of control (uninjected) rats estimated at various times of the day and night. There is an obvious diurnal variation in the mucosal DNA content which is at a maximum between 03:00 and 04:00 and a minimum around 15:00.

Feeding pattern

Fig. 11.14 shows the food intake of rats fed on the Oxoid diet or Diet B observed over a single 24 hour period. When rats were fed on Oxoid and caged in groups of 4, 40% of the total daily food intake was consumed within a 3 hour period, 19:00 - 22:00, which coincided with the start of the dark period (Fig. 11.14b). Throughout the rest of the 24 hours there was a small, but sustained, food intake of about 0.5 g/h/rat. This pattern was observed regardless of whether the

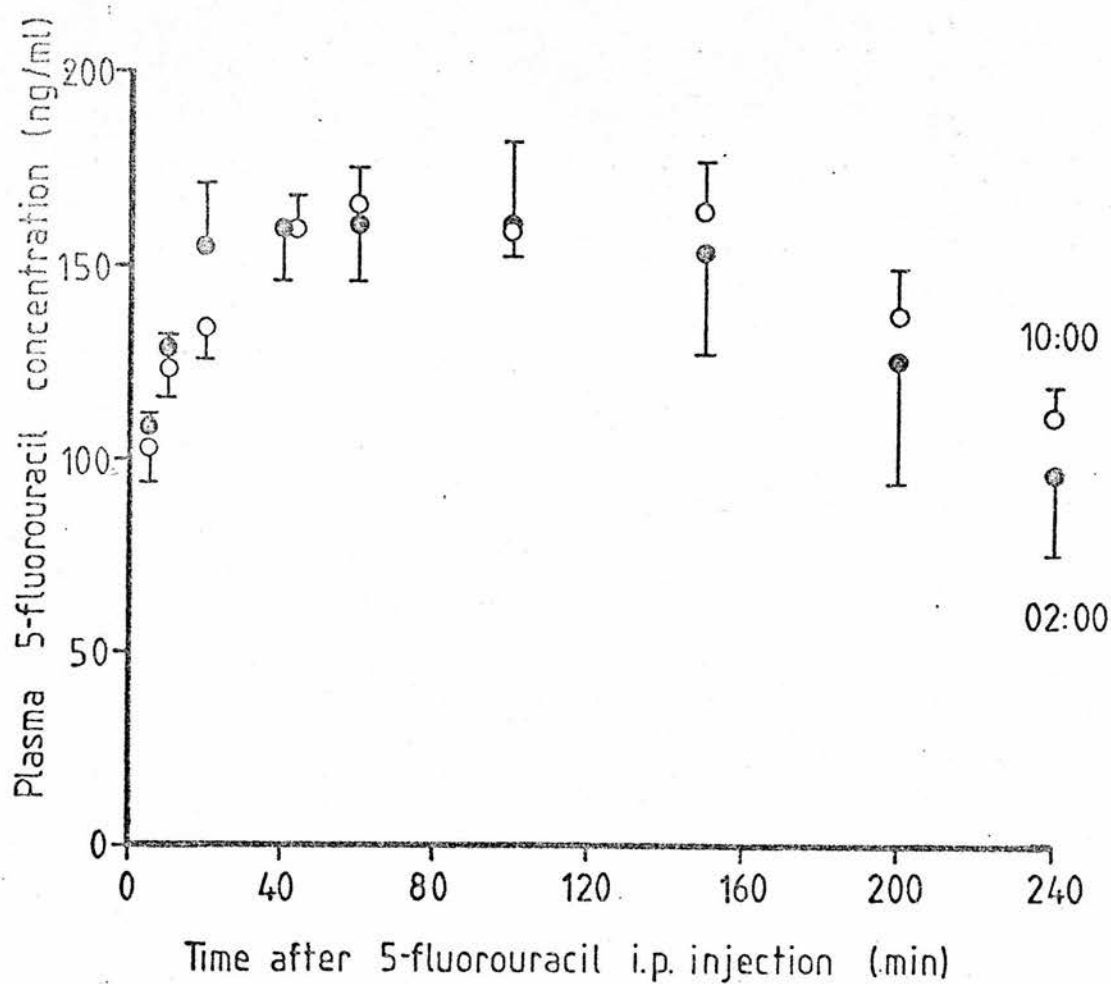


Fig. 11.12 Plasma levels of 5-fluorouracil after a single intra-peritoneal injection at either 10:00 (o) or 02:00 (●) in anaesthetised male rats. Values are the means \pm S.E.M. of observations on 4 animals at each time.

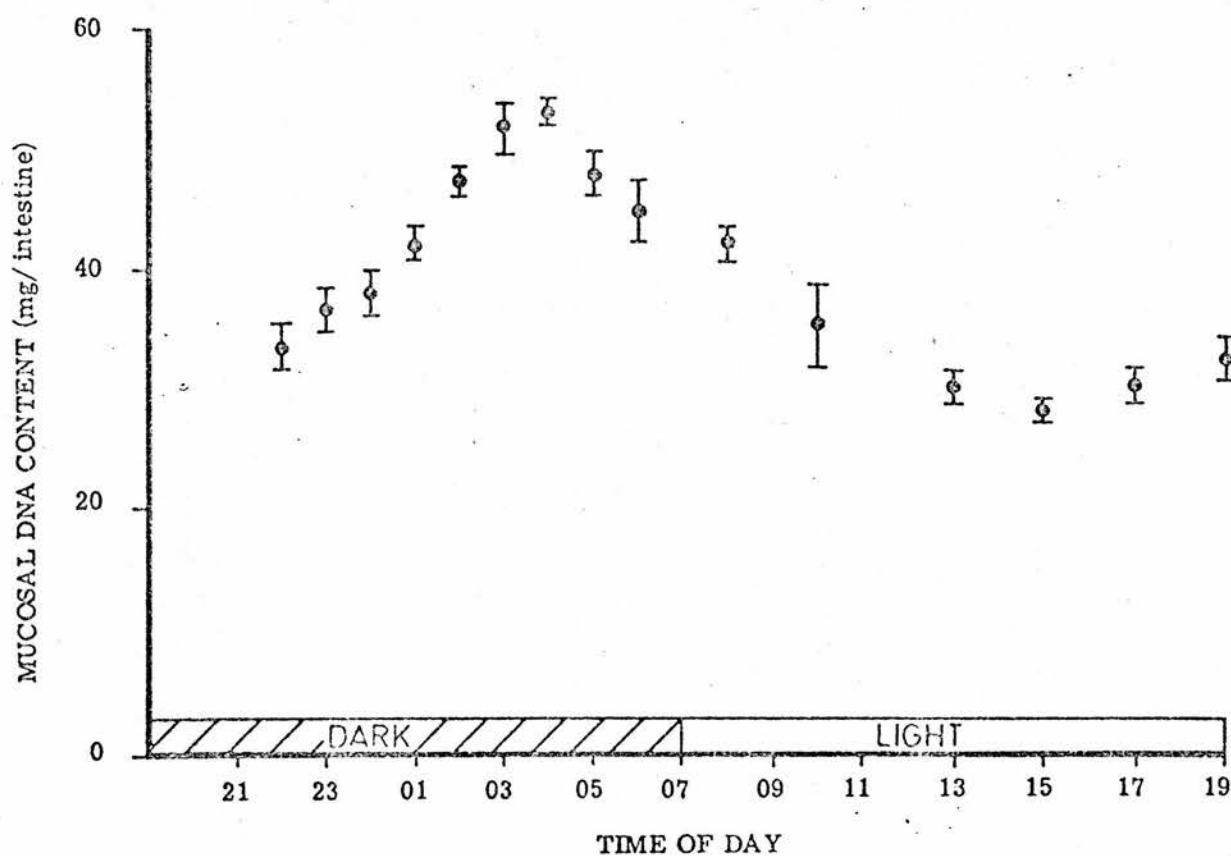
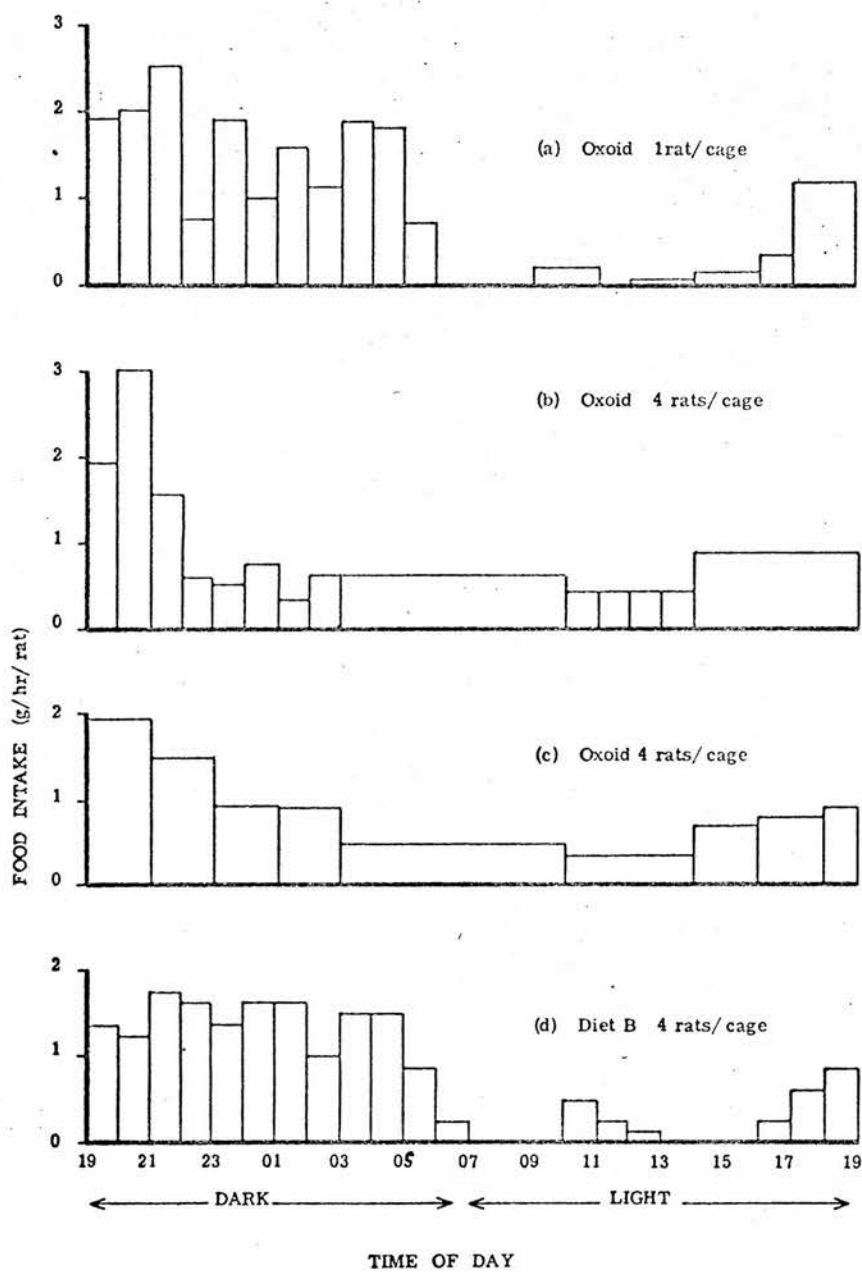


Fig. 11.13 Mucosal DNA content of rat small intestine (whole jejunum plus ileum) estimated at various times of the day and night. Values are the means \pm S.E.M. of estimates from 4 rats. The timing of the light dark cycle is shown on the abscissa.

Fig. 11.14 Food intakes of rats observed over a single 24 hour period. Rats were either fed on the Oxoid diet and (a) caged individually, (b) caged in groups of 4 and the food intake observed at 1 hour intervals, (c) caged in groups of 4 and the food intake observed at 2 hour intervals or (d) rats were fed on Diet B and caged in groups of 4. Values are the mean hourly food intake observed over the period represented by the width of the bars. Where there are no bars shown the food intake was zero. The numbers of animals used were (a) 12 rats, (b) - (d) 2 cages of 4 rats. The timing of the light-dark cycle is shown below the abscissa.



observations were made at 1 or 2 hour intervals (Fig. 11.14b and 14c).

However, when the rats were caged individually a strikingly different pattern of consumption of the Oxoid diet was observed (Fig. 11.14a). There was a sustained food intake of about 1.5 g/h/rat throughout the period from 17:00 to 06:00. During the remaining 11 hours the food intake was negligible (0.7 g/rat). The total daily food intake of rats caged individually (24 g/day/rat) was 26% greater than that of rats caged in groups of 4 (19 g/day/rat).

When rats were fed on the 'elemental' diet, Diet B, and caged in groups of 4 there was a sustained food intake of about 1.5 g/h/rat throughout the period from 17:00 to 06:00 and the food intake during the rest of the 24 hours was negligible (1.3 g/rat) (Fig. 11.14d). Thus this pattern was similar to that observed in the individually caged rats eating the Oxoid diet (Fig. 11.14a).

Relationship between feeding time and the intestinal toxicity of 5-fluorouracil

The relationship between the different feeding times observed under different caging patterns and the intestinal toxicity of 5-fluorouracil was then examined. Fig. 11.15 shows the water absorption rates by the small intestine from uninjected control rats (hatched bars) and of rats 3 days after 5-fluorouracil injection at either 02:00, 03:00 or 10:00. The animals had been caged in groups of 4 and fed ad lib on either the Oxoid diet or Diet B, or had been caged individually with free access to the Oxoid diet for at least 1 week before 5-fluorouracil administration. Also shown are the results from rats which had restricted access either to the Oxoid diet or to Diet B only between 19:00 and 22:00 for 14 days prior to 5-fluorouracil

administration. All the rats had free access to food after 5-fluorouracil injection.

The daily body weight gain of uninjected rats on each of the dietary regimes is also shown in the upper part of Fig. 11.15.

Fig. 11.15 shows that the intestinal toxicity of 5-fluorouracil was much less severe after injection at 02:00 than after injection at 10:00 when rats were fed ad lib on the Oxoid diet and caged in groups of 4 as already shown in Figs. 11.6 and 11.7. Prior to injection these rats consumed 40% of their total daily food intake between 19:00 and 22:00 (Fig. 11.14b). However, the intestinal toxicity of 5-fluorouracil was not reduced by administration at 02:00 when rats were caged individually and fed on the Oxoid diet (Fig. 11.15b). These individually caged rats (unlike those caged in groups of 4) did not show any peak in their food intake between 19:00 and 22:00 - see Fig. 11.14a. Similarly, there was no peak in the food intake between 19:00 and 22:00 when rats were fed on Diet B and caged in groups of 4 (Fig. 11.14d); likewise, the intestinal toxicity of 5-fluorouracil in these animals was not reduced by injection at 02:00 (Fig. 11.15d). However, when access to Diet B was limited to only 3 hours each day (19:00 - 22:00) the intestinal toxicity was reduced by drug administration at 02:00 (Fig. 11.15e). When rats were allowed access to the Oxoid diet during this period (19:00 - 22:00) each day there was no beneficial effect of administration of 5-fluorouracil at 02:00 (Fig. 11.15c). Although the food intake of the rats on this regime was similar to that of rats with free access to the Oxoid diet, the growth rate was only about 65% of that of the rats with free access to the Oxoid diet.

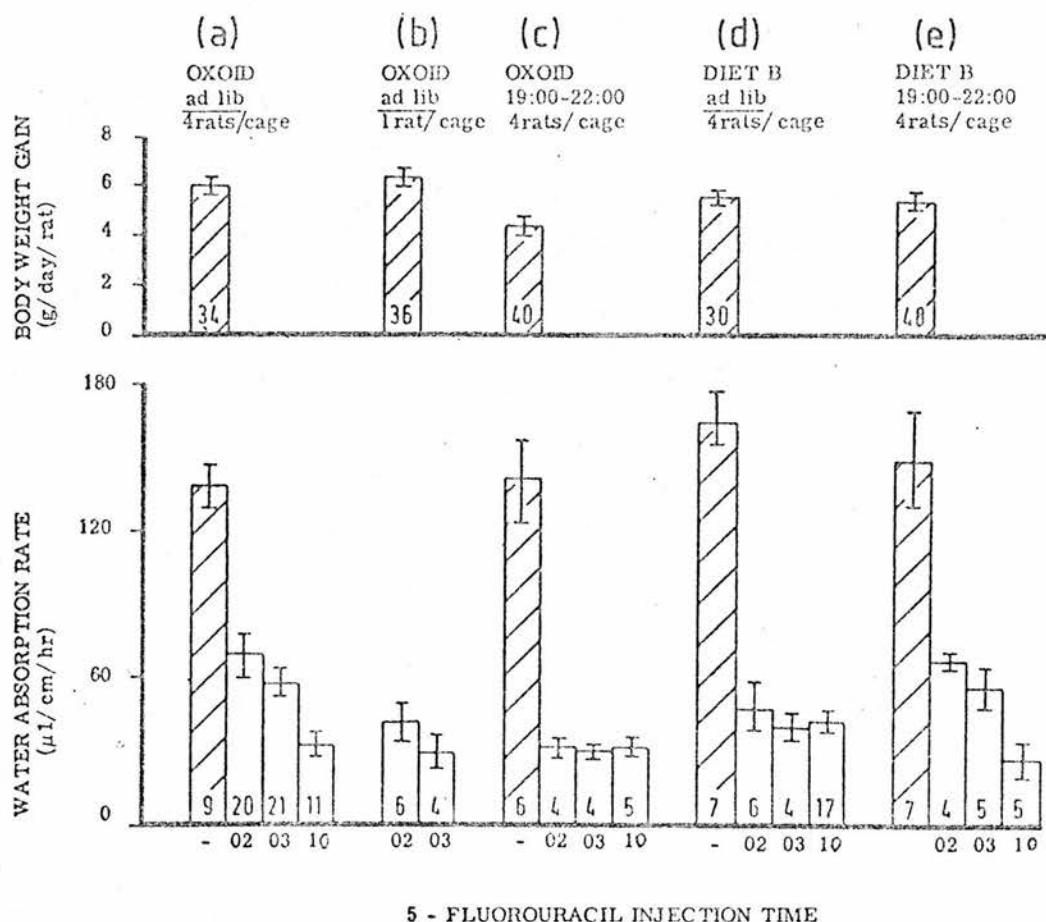


Fig. 11.15 Water absorption rates by the small intestine of control, uninjected, rats (hatched bars) and of rats 3 days after 5-fluorouracil injection at various times. Rats were either caged individually with free access to the Oxoid diet, or were caged in groups of 4 and either had free access to the Oxoid diet or Diet B or had access to the Oxoid diet or Diet B between 19:00 and 22:00 only each day. Values are means \pm S.E.M. and the number of animals used is shown within the bars. The daily weight gain of the rats, prior to 5-fluorouracil injection, on each of the dietary regimes is also shown in the top part of the Figure. Values are means \pm S.E.M. and the number of observations is shown within the bars.

DISCUSSION

The preliminary study, the results of which are shown in Tables 11.1 and 11.2, makes it quite clear that the intestinal toxicity of 5-fluorouracil is influenced by either (1) the feeding time of the animals, (2) the time of drug administration, or (3) the relationship between these two factors. Hence this suggested an obvious line of enquiry which ought to be pursued in any attempt to find conditions under which the toxicity of 5-fluorouracil may be minimised. The experiments in which the feeding time was restricted were not successful. As discussed below these experiments have various weaknesses and it is clear that an unmanageable number of dietary regimes would have to be tested before a definitive assessment of the value of this approach could be obtained. Therefore the approach of altering the time of injection whilst keeping the feeding time constant was pursued and it is this approach which has given the most promising results with a view to minimising the intestinal toxicity of 5-fluorouracil. One of the difficulties in designing sound experiments to test the value of manipulation of 5-fluorouracil injection time is that it is not possible to alter only one of the three possible temporal factors, viz. injection time, time of measurement of absorption, and the interval between these two procedures.

Restriction of feeding time

Although Fisher and Gardner (1976) reported that the growth rate of rats was normal when access to food was limited to only 3 hours each day, others have shown that food restriction results in severely reduced growth rates (Callahan, 1979; Stevenson *et al*, 1975; Stevenson and Fierstein, 1976). The results presented in this study demonstrate

that when access to food is limited, the growth rate of rats can be affected by the diet, the number of animals in each cage and the actual time of day during which access is allowed.

Although the food intake of rats allowed access to the Oxoid diet for only 3 hours each day became very similar by the eighth day of the regime to that of rats fed ad lib, the daily gain in body weight was markedly reduced (Fig. 11.1 and Table 11.3). The decrease was greatest (27%) when rats were fed in the evening only (19:00 - 22:00). This was surprising since rats fed ad lib consume 40% of their total daily food intake during this period (Fig. 11.14b) and it was expected, therefore, that this feeding regime would involve minimal adaptation. A severe reduction of the growth rate of some rats was observed when rats were caged in groups of 5 instead of in groups of 4 (Figs. 11.2 and 11.3); the food intakes of these rats was only 77% of that of rats fed ad lib. It was also noted that rats, fed ad lib on the Oxoid diet, consume 26% more food when caged individually than when caged in groups of 4. These observations may indicate competition between rats for access to food during a short feeding period. Although this suggests that the growth rate of rats fed on the Oxoid diet for only 3 hours each day might have been increased if the rats were caged individually, unfortunately this was not practical due to limited space in the animal house.

However, when rats were fed on the 'elemental' diet, Diet B, the growth rate of those fed for only 3 hours each day was similar to that of rats fed ad lib. Although the growth rate of the latter group was 12% less than that of rats fed ad lib on the Oxoid diet, there is no evidence to suggest that the intestinal toxicity of 5-fluorouracil is

greater in rats fed on this diet. Indeed there is some evidence which indicates that Diet B may reduce the intestinal toxicity of 5-fluorouracil in some rats (see Chapter 10).

Thus it is concluded that a 3 hour feeding period was insufficient to support the normal growth rate of rats fed on an unhydrolysed diet. It may be possible to maintain the growth rate under similar conditions either by housing the rats individually or by extending the feeding period to 4 hours each day. In contrast, when rats are fed on an 'elemental' diet the growth rate and food intakes are unaffected when access to food is limited. Further support for this conclusion is apparent from the decrease in the dry weight of intestines from rats fed for only 3 hours each day on the Oxoid diet (Fig. 11.5). Also, the dry weight of intestines from rats fed ad lib on Diet B was 17% greater than those from rats fed ad lib on the Oxoid diet, and this increase was also apparent in intestines from rats fed for only 3 hours each day on Diet B.

Since it was shown that the intestinal toxicity of 5-fluorouracil is increased by an overnight fast prior to administration of the drug (Table 11.2) these observations indicate that the two feeding regimes (07:00 - 10:00 and 19:00 - 22:00) with the Oxoid diet may increase the intestinal toxicity of 5-fluorouracil. Thus it was of interest to include the two feeding regimes with the 'elemental' diet in this study, since the growth rates and food intakes of rats with restricted access to Diet B were similar to those of rats fed ad lib.

Effects of restricted access to food on intestinal absorption

Previously, Fisher and Gardner (1976) showed that both glucose and

water absorption by the isolated small intestine was 50-60% greater at night than during the day and, furthermore, they showed that this diurnal rhythm was related to the feeding time of the rats. The results presented in Fig. 11.4 are consistent with these observations; water absorption rates by intestines from rats allowed access to the Oxoid diet between 07:00 and 10:00 each day were 59% greater than those from rats fed ad lib on the Oxoid diet (Fig. 11.4). When the Oxoid diet was replaced by Diet B, absorption rates were 20% greater in rats fed ad lib, although this increase was not significant ($P > 0.1$); these rates were also increased by 39% when access to the diet was restricted to the period 07:00 to 10:00. However, absorption rates were unaffected when access to either diet was limited to the period 19:00 to 22:00, which ended at least 12 hours prior to measurement of absorption. This was expected, since this period corresponds to that during which rats fed ad lib on Oxoid consume 40% of their total daily food intake.

Effects of restricted access to food on 5-fluorouracil toxicity

The two feeding regimes were chosen such that 5-fluorouracil was administered immediately after the end of the feeding period (07:00 - 10:00) or 12 hours after the end of the feeding period (19:00 - 22:00). Since intestinal water absorption was impaired to a similar extent in rats fed ad lib on the Oxoid diet and in rats fed for only 3 hours each day on this diet, it is concluded that the intestinal toxicity of 5-fluorouracil is not directly influenced by the presence or absence of food in the gastrointestinal tract, or by the digestive and absorptive activity of the small intestine at the time of drug administration.

No conclusion can be drawn with respect to the effects of feeding

time on the intestinal toxicity of 5-fluorouracil since only two feeding regimes were used. When rats were fed on the Oxoid diet, intestinal water absorption and dry weights were decreased to a similar extent in rats fed ad lib and in rats fed for only 3 hours each day (Fig. 11.4 and 11.5). However, when rats had limited access to Diet B the slight and equivocal benefit, observed in rats fed ad lib on this diet prior to 5-fluorouracil administration, was no longer observed. Since the cause of the decrease in the severity of the toxicity of 5-fluorouracil in some rats fed on Diet B is not known (see Chapter 10), the effects of the feeding regimes cannot be explained.

These results do not exclude the possibility that a relationship exists between feeding time and drug toxicity. However, in order to obtain sufficient information to enable firm conclusions to be drawn a large, and unmanageable, number of feeding regimes would have to be used. A further problem associated both with this approach and with that of varying the drug administration time arises because the quantity of drug administered is calculated on a body weight basis. Consequently a larger dose of 5-fluorouracil (about 12% greater) was administered to rats injected during or just after the feeding period than was administered to rats injected some time after the end of the feeding period. However, it was shown in Chapter 8 (Fig. 8.8) that the intestinal toxicity of 5-fluorouracil was equally severe when the dose was reduced by 25%.

The results obtained with the Oxoid diet indicate that these feeding regimes do not maintain the normal growth of rats. It is clear that this approach has weaknesses and therefore it was not pursued further in favour of the approach of variation of the drug

administration time.

Variation of drug administration time

The alternative of varying the time of 5-fluorouracil administration has proved to be the most promising approach to the problem of reduction of the toxicity of this drug. The results in Figs. 11.6 and 11.7 show clearly that the intestinal toxicity of 5-fluorouracil varies markedly with the time of administration of the drug. Least toxicity, estimated both by the effects on intestinal water absorption in vitro and by the incidence of diarrhoea in vivo, is observed in rats which have been injected with the antimetabolite at 02:00 (Figs. 11.6 and 11.7 and Plate 11.1). The duration of intestinal toxicity is, however, independent of the injection time (Fig. 11.9). Also, although the time of injection did not affect the food intakes of the rats, the reduction in body weight by the fifth day was less after injection at 02:00 (Figs. 11.8 and 11.10).

Absorption rates were significantly greater ($P < 0.01$) by a factor of 3 after administration at 02:00 than after administration at 01:00. Since both of these estimates were made on the third day after 5-fluorouracil administration, it is clear that the diurnal variation is not the result of estimation of absorption rates at varying times between 3 and 4 days after 5-fluorouracil administration. Also, since the minimum toxicity was observed in rats injected just after the peak of feeding activity (19:00 - 22:00), the diurnal variation in toxicity cannot be the result of drug administration on a body weight basis. Furthermore, apart from the rise on the first day after administration of 5-fluorouracil at 15:00, the injection time associated with maximal intestinal toxicity, absorption rates were consistently higher after

administration at 02:00 than after administration at 15:00 on each of the first 5 days after administration of 5-fluorouracil (Fig. 11.9). Although absorption rates were not significantly increased on the first day after administration at 10:00 (Chapter 8), Gardner et al (1978) demonstrated a significant increase at this time. It is possible that there is a transient increase in absorption rates on the first day after injection of the drug and that this was not observed after administration at 02:00, since the first measurements of absorption were not made until 32 hours after drug administration.

The plasma pharmacokinetics of 5-fluorouracil were similar after intraperitoneal administration at 10:00 and at 02:00 (Fig. 11.12) and this suggests that the differential toxicity of 5-fluorouracil after administration at various times is not due to a major difference in absorption and metabolism of the drug at these times.

In these experiments, 5-fluorouracil was administered via the intraperitoneal route and the observations on plasma pharmacokinetics, discussed in Chapter 9, indicate that plasma levels of the drug remain elevated for at least 3 to 4 hours - see also Fig. 11.12. Thus a single intraperitoneal injection at 02:00 is equivalent to an intra-arterial infusion sustained over the period 02:00 to 06:00 (Fig. 11.12). This raises the possibility that the toxicity might be decreased even further by administration via a single bolus intra-arterial or intravenous injection - a possibility which merits further attention.

There is a diurnal variation in intestinal cell proliferation and in the number of villus cells (Al-Dewachi et al, 1976; Stevenson et al, 1979). Al-Dewachi et al (1976) showed that in the rat jejunum the mitotic index was minimal at 02:00 and maximal at 15:00, and this is

consistent with the observation of a diurnal variation in the mucosal DNA content which is maximal between 03:00 and 04:00 and minimal around 15:00 (Fig. 11.13). Hence the optimum time for 5-fluorouracil administration may coincide with the maximum number of cells in the post mitotic resting (G_1) phase or simply with the maximum number of intestinal cells.

Halberg (1977) demonstrated that cytosine arabinoside, when injected on a sinusoidal schedule, is less toxic to host tissue in mice when the largest portion of the total 24 hour dose is given around the mid-point of the light span. This is consistent with the results of this study, since the diurnal rhythm of proliferative activity in the mouse small intestine is 12 hours out of phase with that of the rat (Sigdestad and Leshner, 1970).

The substantial diurnal variation in mucosal DNA content (Fig. 11.3) corresponds approximately to the diurnal variation in glucose and water absorption already observed (Fisher and Gardner, 1976). This suggests that the increase in absorption rates at night could be due to an increase in the number of absorbing cells. However, it must be noted that the mucosal DNA content includes cells from the crypts and from the lamina propria as well as those of the villus and therefore is not a specific index of the number of absorbing cells. Stevenson et al (1979) showed that there is a diurnal variation in both the villus height and the number of villus cells and that both are maximal around the time of feeding. Their results lend support to the above suggestion.

Diurnal variations in various intestinal absorptive and enzymic activities and in the proliferative state of the epithelium have been

shown to be related to the feeding time in rats (Fisher and Gardner, 1976; Stevenson et al, 1975; Stevenson and Fierstein, 1976). Therefore an attempt was made to relate the optimal injection time to the feeding habits of the rats.

Two markedly different feeding patterns were observed depending on both the diet and the number of rats in each cage. When rats were caged in groups of 4 and fed on the Oxoid diet, there was a peak of food intake during the period 19:00 - 22:00, and a small but sustained food intake throughout the rest of the 24 hours (Fig. 11.14b); a pattern similar to that observed elsewhere (Kimura et al, 1970; Vonk et al, 1978). Administration of 5-fluorouracil at 02:00 was associated with a reduction in the intestinal toxicity of the drug only when a major portion of the daily food intake occurred in the period 19:00 - 22:00 (Figs. 11.14 and 11.15). This pattern was observed either (a) when rats were caged in groups of 4 and had free access to the Oxoid diet or (b) when access to the 'elemental' diet, Diet B, was restricted to the period 19:00 - 22:00 daily (Fig. 11.14). In contrast, when rats had free access to Diet B or when they were caged individually with free access to the Oxoid diet, there was no peak of food intake and no benefit was apparent from administration of 5-fluorouracil at 02:00 (Figs. 11.14 and 11.15). Although a reduction in the intestinal toxicity of 5-fluorouracil was not observed after administration at 02:00 to rats that had been fed on the Oxoid diet only between 19:00 and 22:00 each day it should be noted that the growth rate of rats on this dietary regime was relatively low (Table 11.3 and Fig. 11.6). This was discussed earlier and these observations lend further support to the conclusion that the restricted feeding regimes with the Oxoid diet do not support the normal growth rate of rats, whereas those with

the 'elemental' diet are comparable to ad lib regimes.

Thus, the intestinal toxicity of 5-fluorouracil does depend on the injection time and the optimal injection time appears to be influenced by the feeding time, although it cannot be concluded unequivocally that the two are directly related.

Exploitation of these observations is clearly of potential value to enhance the therapeutic value of 5-fluorouracil and, possibly, of other cytotoxic agents and of irradiation provided that tumour tissue does not exhibit the same diurnal rhythm in sensitivity. Further studies on the relationship between the optimal injection time and the feeding time and on cell kinetics should be useful in predicting and manipulating the optimum time for 5-fluorouracil administration to humans. Even if target and host cells do exhibit a similar rhythm in toxicity, such studies might well provide a rational basis for dissociating the rhythms so as to obtain maximum effect on a tumour while minimising the host side effects, especially gastrointestinal ones. Certainly if an optimal time for 5-fluorouracil administration to humans was established and confirmed, the exploitation of this 'chronopharmacological' approach would have much to commend it, including its simplicity.

CHAPTER 12

General Discussion

The aims of this study have, on the whole, been achieved. The results extend previous studies of the effects of 5-fluorouracil on the small intestine (Gardner et al, 1978; Levin, 1968; Roche et al, 1970) and now demonstrate clearly the dissociation of the effects of 5-fluorouracil on the small intestine from those simply consequent to a reduced food intake. Although Bounous et al (1971a and b) claimed that an 'elemental' diet could reduce the systemic toxicity of 5-fluorouracil, the results of this study do not support this claim. On the contrary, the results suggest that these diets might even increase the systemic toxicity of the drug. However, the most rewarding part of this work was the identification of a possible means of reducing the intestinal toxicity of 5-fluorouracil, namely selection of an "optimal" time of day for 5-fluorouracil injection.

The results have been discussed in detail in each chapter and only the main points will be discussed in this final chapter.

The methods

The results, in retrospect, confirm that the methods chosen for the study of intestinal absorption, cytoplasmic peptide hydrolase activities and mucosal DNA contents, were sound. All three methods gave consistent and reproducible results throughout this work and the values obtained compare well with those reported in the literature.

In Chapter 3 it was stressed that one of the major problems associated with the development of intestinal preparations in vitro

was that of supplying sufficient oxygen to the mucosal cells. A possible result of neglect of this factor is shown clearly in Chapter 6. The amount of peptide hydrolase activities released from the small intestine during perfusion in vitro of intestines removed, after establishment of perfusion, from anaesthetized rats is normally small and very similar to that observed with perfusion in vivo (Fig. 6.6). However, if the animal is killed immediately prior to the establishment of luminal perfusion, a dramatic increase in the amount of peptide hydrolase activity released is observed (Fig. 6.6). Furthermore, Gardner (1978) showed that this also results in a 50% reduction in the water absorption rate. Although it cannot be concluded that these changes are a direct result of temporary anoxia, it must be stressed that intestines removed from freshly killed animals are frequently used for other intestinal preparations in vitro such as everted sacs (e.g. Wilson and Wiseman, 1954) and tissue rings (e.g. Agar et al, 1956). These observations seriously question the use of these preparations for the study of intestinal absorption. This applies especially to studies on peptide absorption and on peptide hormones since these preparations may release considerable amounts of peptidase activities into the incubation medium.

These observations show the advantages of the method of Fisher and Gardner (1974), chosen for the measurement of water absorption in this work, provided that their precautions to avoid temporary hypoxia are observed. They also show, for the first time, that observations such as those of Silk and Kim (1976) on enzyme release from intestinal preparations in vitro are strictly dependent on details of experimental technique, a point which is regrettably neglected in many laboratories.

The intestinal toxicity of 5-fluorouracil

Gardner *et al* (1978) concluded that malabsorption may account for some of the undesirable side effects associated with the administration of 5-fluorouracil. The results of this study agree with their conclusion although, of course, no consideration has been given to the effect on colonic function which is likely to be involved in the pathogenesis of diarrhoea.

Intestinal absorption rates were unaffected 24 hours after drug administration but were decreased to only 26% of control rates by the third day. A similar decrease was observed on the fourth day but by the fifth day after drug administration absorption rates had recovered to about 70% of control rates (Fig. 8.3). Since the turnover time of the epithelium of the rat small intestine is between two and three days (Eastwood, 1977) these changes in absorption rates could be explained simply by an inhibition of crypt cell production by 5-fluorouracil and a consequent loss of cells to replace the mature enterocytes extruded from the tips of the villi. This theory is supported by the observation that the marked decrease in the mucosal DNA content 24 hours after drug administration (Fig. 8.10) does not result in an immediate reduction in water absorption rates.

The results presented in Chapter 8 are entirely consistent with the theory that the impairment of intestinal absorption observed after 5-fluorouracil administration is the result of a decrease in the number of absorbing cells, as suggested by Levin (1968), rather than a decrease in the absorptive capacity of individual absorbing cells. However, this question cannot be resolved conclusively, since there is no method available to determine the number of

absorbing cells in the intestinal mucosa. Whilst the mucosal DNA content is a useful index of the total number of mucosal cells and of the proliferative state of the intestinal epithelium, it does not distinguish between different cell types and is therefore not a specific index of absorbing cells as implied by Batt and Peters (1976). Furthermore, the results shown in Figs. 8.11 and 8.12 demonstrate the importance of the choice of reference system for the expression of absorption rates, especially when studying the effects of an antimetabolite on an heterogeneous population of cells such as the intestinal mucosa. It is concluded that absorption rates should be expressed per whole small intestine whenever possible especially since these estimates are associated with the least variance of the four reference systems used; they should reflect the overall consequences for the animal.

While there is a dramatic decrease in the intestinal water absorption rate and mucosal DNA content after 5-fluorouracil administration the consequent reduced food intake alone results in a much smaller decrease (Figs. 8.11 and 8.12). It is clear, therefore, that the effects of 5-fluorouracil on the small intestine cannot wholly be attributed to a reduction in food intake. This conclusion is further supported by the observation that the Tris and sodium salts of 5-fluorouracil have markedly different effects on the food intake of rats (Fig. 8.1) but have very similar effects on the small intestine (Figs. 8.3 and 8.4).

The Tris and sodium salts of 5-fluorouracil

The introduction of the sodium salt of 5-fluorouracil was fortuitous to the dissociation of the effects of 5-fluorouracil per

se on the small intestine from those of the reduced food intake. However, the dramatic difference in the systemic toxicities of the Tris and sodium salts of the drug was both unexpected and the cause of some concern. A 16% mortality was observed following administration of the Tris salt of 5-fluorouracil and all of the rats injected with this salt of the drug showed signs of severe systemic toxicity within three to four hours after injection. In contrast, no such toxicity and no deaths were recorded following administration of an equimolar dose of the sodium salt of 5-fluorouracil. There is a difference between the plasma pharmacokinetics of the two salt forms but this is only apparent after intraperitoneal injection (see Chapter 9). Plasma 5-fluorouracil concentrations rose to and were sustained at a higher level after administration of the Tris salt than after administration of the sodium salt of 5-fluorouracil. This difference alone is not thought to be sufficient to explain the severe systemic toxicity associated only with administration of the Tris salt of the drug but is thought to be indicative of a difference in the properties of the two salt forms. It is therefore suggested that it is a property of the Tris salt per se that is responsible for the severe toxicity and mortality associated with its use. Whether this difference in the properties of the two salt forms of the drug is reflected in their ability to kill tumour cells is not known. The plasma pharmacokinetic studies suggest that there may be a difference in the bioavailability of the Tris and sodium salts of 5-fluorouracil. However, it is difficult, in retrospect, to determine whether tumour response to chemotherapy with 5-fluorouracil is affected by the salt form of the drug used or indeed whether the toxicity differs since authors seldom state the salt form of a drug used. A number of reports of cardiotoxicity

following 5-fluorouracil administration (see review by Villani et al, 1979) were published during the period when the Tris salt was available on the U.K. market and it would be interesting to know if the incidence of adverse effects has decreased since the introduction (November 1978) of the sodium salt. The records of adverse reactions to drugs held by the Committee on Safety of Medicines shed no light on this point (M.L.G. Gardner, Personal Communication).

It is unlikely that the difference in the systemic toxicities of the two salt forms of 5-fluorouracil could be exploited in an attempt to reduce the clinical toxicity of the drug since the intestinal toxicity of the two salt forms is very similar and this toxicity is one of the major causes of the termination of chemotherapy with the drug. However, the difference between the Tris and sodium salts of 5-fluorouracil clearly merits further attention and reinforces the principle that seemingly minor changes in drug formulation can have important biological consequences.

'Elemental' diets

The rationale behind the claim by Bounous et al (1971a and b) that an 'elemental' diet could reduce the systemic toxicity of 5-fluorouracil seemed reasonable and therefore this possibility was investigated in detail. However, the results presented in Chapter 10 do not support this claim. These diets do not maintain the normal growth rate observed in rats fed on a standard unhydrolysed diet, although the growth rates of rats fed on one particular 'elemental' diet were increased to a rate comparable to that of animals fed on a normal diet by the novel use of dietary flavouring (Table 10.7). Furthermore, the energy utilization from these diets is very

inefficient and this suggests that these diets are not ideally suited to the nutrition of rats. If this is true for humans then these diets do not meet the stated requirement - "to reduce each substrate to the most efficient unit for digestion, absorption, transport and utilization" (Tweedle, 1976). These observations alone argue against the use of 'elemental' diets in patients with malabsorption as a consequence of tumour growth (Costa, 1977).

If body weight loss after 5-fluorouracil administration is regarded as a criterion of toxicity then it must be concluded that 'Flexical' and 'Vivonex-HN' increase the systemic toxicity of 5-fluorouracil (Fig. 10.2). Furthermore, none of the 'elemental' diets reduced the severe impairment of intestinal absorption observed after 5-fluorouracil administration (Fig. 10.6). It is noteworthy that the most recent reports from Bounous and associates (Bounous *et al*, 1978a and b) and an independent group of workers (Stanford *et al*, 1977) have eventually reported that 'elemental' diets actually increase the mortality observed after 5-fluorouracil administration; this must argue against their use regardless of the earlier claims of benefit to the intestine. As explained in Chapter 10, the only evidence in support of their earlier claim, relating to the measurement of intestinal absorption, was provided by Mohiuddin and Kramer (1978), and this did not stand up to critical scrutiny.

The mucosal DNA content is decreased to a similar extent after administration of 5-fluorouracil to rats fed on a standard diet and to rats fed on an 'elemental' diet (Fig. 10.4). Since it is concluded that the impairment of intestinal absorption is probably due to a decrease in the number of absorbing cells, it is clear that 'elemental' diets do not have a prophylactic action and the results

of this study suggest that they do not aid the recovery of the intestine from the injury following 5-fluorouracil administration. It must therefore be concluded that 'elemental' diets do not reduce the intestinal toxicity of 5-fluorouracil.

Time of drug administration

By far the most rewarding part of this study was the observation that the intestinal toxicity of 5-fluorouracil varies with the time of drug administration. Least toxicity, estimated both by the effects on water absorption in vitro and by the incidence of diarrhoea in vivo, was observed following injection at 02:00 and maximum toxicity was observed following injection at 15:00 (Figs. 11.6 and 11.9). These two times coincide with the nadir and peak respectively of the diurnal rhythm of proliferative activity in the rat small intestine (Al-Dewachi et al, 1976). This suggests that the intestinal toxicity of 5-fluorouracil is related to the proliferative activity of the mucosa at the time of administration and this possibility is consistent with the observation that the mucosal DNA content is maximal at around 03:00 and minimal at around 15:00 (Fig. 11.13). Furthermore, it has already been shown that 5-fluorouracil is more toxic to proliferating cells than to non-proliferating cells (Bruce and Meeker, 1967).

These observations suggest a simple means of reducing the intestinal toxicity of 5-fluorouracil, i.e. by careful selection of the time of drug administration. However, in order to apply this method to the clinical use of 5-fluorouracil, the time of minimal proliferative activity in the human small intestine must be identified. Unfortunately little is known about the proliferative

activity of the human small intestine since ethical considerations prevent the use of the most useful methods of cell kinetic analysis such as the labelling index and metaphase arrest techniques (Aherne et al, 1977, p. 68). However, it may be possible to identify the time of minimal intestinal proliferative activity by counting villus populations or by measuring villus height in biopsies taken from the same region of the small intestine at various times of the day and night. Certainly the benefits to be gained from a reduction in the systemic toxicity of 5-fluorouracil and of other chemotherapeutic agents justifies further investigation of this 'chronopharmacological' approach: it has much to recommend it, especially its simplicity and lack of extra expense (c.f. the use of 'Vivonex-HN' is estimated at £16.60 per patient per day by Eaton Laboratories Ltd). It may be necessary and possible to synchronise or cue the timing of the cell cycle (e.g. by dietary manipulation); this might make it possible to predict accurately the optimal time for 5-fluorouracil injection in humans and to schedule this for a 'convenient' time.

A major shortcoming of this work is that haematological toxicity has not been considered. This exclusion is justified, in part, since the gastrointestinal toxicity of 5-fluorouracil precedes the haematological toxicity (Calabresi and Parks, 1975) by about four days and therefore the same animals could not be used for both studies. However, Halberg et al (1973) showed that there is a diurnal variation in the proliferative activity of the bone marrow of rats and that the activity is minimal at around 00:00. Thus it is possible that the optimum time of injection in order to minimise the gastrointestinal toxicity of 5-fluorouracil may well coincide with that producing minimal haematological toxicity.

The plasma pharmacokinetics of 5-fluorouracil showed (Fig. 9.5) that an intraperitoneal injection is equivalent to an intravenous infusion sustained over a three to four hour period; this raises the possibility that even less intestinal toxicity might be observed if the drug were administered at the optimum time as a single bolus intravenous injection. This merits urgent consideration.

Thus the work reported in this thesis suggests that practical improvements in chemotherapy may be simply achieved. More work is clearly required and it is greatly hoped that the present findings will stimulate interest in the 'chronopharmacological' approach to chemotherapy.

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The series resistance determined in this manner is higher than the usually measured resistance of the bathing solution before mounting the tissue. This is probably due to the contribution of the resistance of the subepithelial tissue layers to R_s . Therefore the true epithelial resistance is lower than that measured with the conventional method (table).

The short circuit current increases because of the decrease in R_s . Consequently, as was shown in rabbit ileum, the sodium net transfer rises from 5.74 ± 0.75 (SEM, $n = 17$, R_s measured conventionally) to 8.62 ± 0.92 (SEM, $n = 17$, R_s measured with the new method).

Vascular perfusion of the rat small intestine.

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A vascularly perfused preparation of the rat small intestine, based on the method of Hanson and Parsons (1), will be described. In this preparation, the lumen of the jejunum is cannulated and perfused with Krebs-Henseleit bicarbonate medium. The flow of luminal perfusate is interrupted with bubbles of 95 p. 100 O_2 , 5 p. 100 CO_2 (segmented circulation) which gives increased stirring in the lumen and improved oxygenation of the epithelial tissues. The vascular system serving the jejunum is also perfused with Krebs-Henseleit buffer, supplemented with 2.5 p. 100 bovine serum albumin and washed red cells at an hematocrit of 25 p. 100, which is introduced via the superior mesenteric artery and collected from a cannula inserted into the hepatic portal vein.

This method offers considerable advantages over more conventional *in vitro* preparations such as isolated loops, gut rings or everted sacs, since the tissue oxygenation is improved, the vascular flow prevents the accumulation of substances taken up from the lumen to unphysiologically high concentrations in the epithelial cells and the metabolism of nutrients can be studied as well as their transport. Thus, the vascularly perfused preparation comes close to duplicating the *in vivo* situation while still retaining much of the versatility of measurement afforded by other *in vitro* systems.

Measurements of various parameters of the preparation such as sugar uptake and lactate output will be presented, together with a consideration of the problems and technical details encountered in setting up such a system.

1. HANSON PJ, PARSONS DS : *J Physiol (Lond)*, 1976, 255, 775.

The physiological viability of isolated intestine prepared from dead animals. J. A. PLUMB and M. L. G. GARDNER (Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U. K.).

Many intestinal preparations are set up after the animal is killed and the intestine removed. However

even if this is rapidly performed damage may occur.

Glucose and water absorption in perfused small intestine are halved if the intestine is rapidly removed from a dead rat instead of being connected to the apparatus while the animal is under anaesthesia (2). Also, rates of leakage of amino acids and cytoplasmic dipeptide hydrolases from the intestine into the luminal perfusate are substantially higher when the rats are killed by ether than when the animals are kept alive under ether anaesthesia until after the flow of oxygenated perfusate has been established through the lumen (fig.).

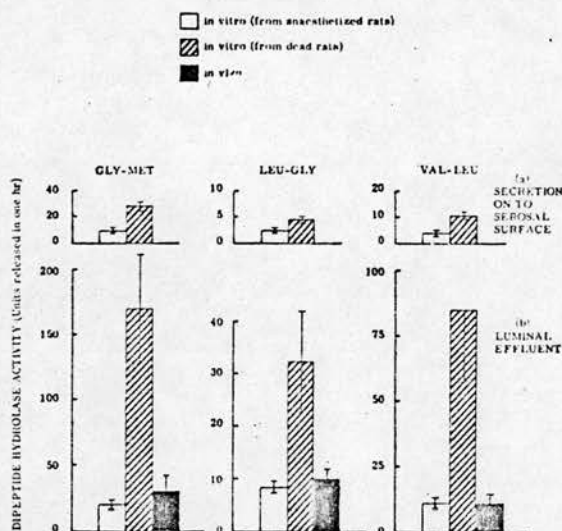


FIG. — Rates of release of hydrolase activities against gly-L-met, L-leu-gly and L-val-L-leu into (a) serosal secretion and (b) luminal effluent during luminal perfusion *in vitro* and *in vivo*.

These findings suggest that the precautions of Fisher and Gardner (1, 2) to avoid temporary hypoxia should be adopted whenever possible. Neglect of this may (a) decrease absorption, (b) increase experimental variability and (c) permit peptide hydrolysis *in vitro* at an unphysiological site (3).

1. FISHER RB, GARDNER MLG : *J Physiol (Lond)*, 1974, 241, 211.
2. GARDNER MLG : *Q J Exp Physiol*, 1978, 63, 95.
3. SILK DBA, KIM YS : *J Physiol (Lond)*, 1976, 258, 489.

Kinetic study of D-galactose and D-mannitol fluxes across a doubly-perfused intestinal segment, *in vitro*. J. BATAILLE (Laboratoire de Physiologie Comparée, Orsay, France).

In order to study the transmucosal fluxes of sugars across the intestinal wall, an isolated segment of guinea-pig jejunum was perfused on serosal and mucosal sides (1.5 ml/min). After 30 min equilibra-

The intestinal toxicity of an antimetabolite depends upon the hour of injection

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Although antimetabolites are widely used in chemotherapy their side effects, especially intestinal ones, can be severe. The therapeutic use of antimetabolites could be extended if this toxicity were reduced. We have previously shown direct impairment of intestinal absorption by 5-fluorouracil (5FU)(1) and have now attempted to minimise this by (a) manipulating the time of injection of 5FU and (b) feeding 'elemental' diets as suggested by Bounous et al (2).

Rats were injected i.p. with 5FU at various times. Subsequently water absorption rates were measured in vitro in the perfused small intestine (3). The incidence of diarrhoea was recorded.

Water absorption rates varied with the hour of injection: the highest rates were observed in rats injected at 3 a.m. The least incidence of diarrhoea also occurred after injection at 3 and 4 a.m.

The 'elemental' diet "Vivonex" or diets based on hydrolysed lactalbumin or casein do not reduce the intestinal toxicity(4). We have now shown that the 'elemental' diet "Flexical" and a diet based on casein hydrolysate, sucrose and oil as used by Bounous et al (2) also fail to protect intestinal absorption from 5FU toxicity.

We conclude that: (a) Manipulation of drug injection time offers a potential means of increasing the therapeutic value of 5FU and (b) 'Elemental' diets do not provide direct protection of the intestine from 5FU toxicity.

- (1) Gardner, MLG, Samson, RR, Heading, RC: Clin.Sci., 1978, 54, 411.
- (2) Bounous G, Gentile, JM, Hugon, J: Can.J.Sur., 1971, 14, 298
- (3) Fisher, RB, Gardner, MLG: J. Physiol (Lond)., 1974, 241, 211.
- (4) Gardner, MLG, Heading, RC: Clin.Sci., 1971, 56, 243.

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SHORT COMMUNICATION

DIFFERENTIAL TOXICITY AND PHARMACOKINETICS OF SODIUM AND TRIS
SALTS OF 5-FLUOROURACIL IN THE RAT

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Summary

1. Rats injected intraperitoneally (i.p.) with the Tris salt of 5-fluorouracil (1.44 mmol/kg) showed severe toxic symptoms and a 15% mortality. No mortality and no obvious toxicity, apart from intestinal toxicity, resulted after injection of the sodium salt at equimolar dosage.
2. The plasma pharmacokinetics of the two salts of 5-fluorouracil (1.44 mmol/kg) were different after i.p. injection: higher plasma levels were achieved and were sustained for longer after the Tris salt than after the sodium salt.
3. No differences in plasma pharmacokinetics were observed after intra-arterial (i.a.) injection of the two salts of 5-fluorouracil (0.576 mmol/kg).
4. Thus there are fundamental (and potentially important) differences between the biological properties of the sodium and Tris salts of 5-fluorouracil. These cannot be explained at present.

Key words: 5-fluorouracil, pharmacokinetics.

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Introduction

In the course of our investigations into the effects of 5-fluorouracil on intestinal absorption (Gardner, Samson & Heading, 1978; Gardner & Heading, 1979; Gardner & Plumb, 1981) we consistently observed toxic symptoms and an appreciable mortality when rats were injected with a high dose (1.44 mmol/kg) of the Tris salt of 5-fluorouracil but not after an equimolar dose of the sodium salt. From analysis of animals' food intakes, growth rates, mortality and signs of gross pancreatic lesions it was clear that the effects of the two salts of the drug were highly significantly different, although their direct effects on intestinal absorption were closely similar (Gardner & Plumb, 1981). Since there was no obvious reason for this difference we have now measured the plasma pharmacokinetics of the two salts of 5-fluorouracil after intraperitoneal (i.p.) or intra-arterial (i.a.) injection.

Materials and Methods

Observation on toxicity

Male and female Wistar rats of a local strain, weighing 160-200 g, were allowed free access to Oxoid 'Diet 86' (Oxoid Ltd, Basingstoke, Hants, U.K.) and water. They were injected i.p. with a sterile aqueous solution of 5-fluorouracil (1.44 mmol/kg) as either the Tris salt (384 μ mol/ml) or the sodium salt (192 μ mol/ml) while temporarily under light ether anaesthesia. The 5-fluorouracil solutions and a matched Tris placebo were kindly provided by Roche Products Ltd, Welwyn Garden City, Herts. U.K. All injections were given between 10:00 and 10:30 hours.

Measurement of plasma pharmacokinetics

Female Wistar rats of our local strain, weighing 195-205 g, were used. They had free access to Oxoid 'Diet 86' and water until the time of the experiment. Carotid arterial blood (0.3 ml per sample) was withdrawn via an indwelling cannula in urethane-anaesthetized rats (a) over the first 240 min after i.p. injection of 5-fluorouracil, sodium or Tris salt (1.44 mmol/kg as in the other experiments described above) through a cannula inserted through a laparotomy so that variability in the exact site of injection could be minimized, and (b) over the first 60 min after intra-carotid arterial injection of 5-fluorouracil, sodium or Tris salt (0.576 mmol/kg). The Tris salt as provided by Roche Products Ltd was diluted 1:1 with the matched Tris placebo for these experiments on pharmacokinetics so that both salts were given at the same concentration, 192 μ mol/ml. Fifteen rats were

injected with each salt by the i.p. route; 3 with sodium salt and 4 with Tris salt by the i.a. route.

5-Fluorouracil was determined by the absorbance at 267 nm after ultrafiltration of the plasma, diluted 10-fold with potassium phosphate buffer (0.1 mol/l, pH 7.0), through an 'Ultrafree' filter (Millipore (U.K.) Ltd, London). Control plasma treated in this way showed no absorbance at 267 nm; the recovery of 5-fluorouracil added to control plasma was $101 \pm 3.3\%$ (n=5) in the range 50 - 250 ng/ml. Nevertheless it should be noted that this assay is not specific for 5-fluorouracil, and that metabolites of the drug probably also contribute to the absorbance. The limit of detection was approximately 20 ng/ml.

Results

Observations on toxicity

Within about 3-6 h after i.p. injection of the Tris salt of 5-fluorouracil all rats became cold and very lethargic with obvious respiratory distress. In some cases death resulted, although we do not know the actual cause of death. Severe macroscopic haemorrhagic lesions on the surface of the pancreas were noted. After injecting 319 rats with the Tris salt we have observed a 15% mortality; 27 out of 142 males and 21 out of 177 females died. The food intake of some of these animals was reported by Gardner & Plumb (1981); it was negligible during the first 48 h after 5-fluorouracil injection, and then it progressively returned to normal over the following 7 days.

In contrast, after injecting 449 rats (436 males and 13 females) with the sodium salt at the same molar dosage we have not recorded any deaths; furthermore we have never observed any signs, apart from diarrhoea and impaired intestinal absorption, of the systemic toxicity that was observed after injection of the Tris salt. Also, the food intake, reported by Gardner & Plumb (1981), over the first 48 h after injection with the sodium salt was decreased to only 50% of the pre-injection intake; it continued to decrease over the next few days before increasing towards normal, a pattern which markedly differed from that after administration of the Tris salt (Gardner & Plumb, 1981).

The toxicity and mortality associated with the Tris salt were not reduced when the 5-fluorouracil was diluted 1:1 with water or with Tris placebo so that the concentration and

osmolarity, respectively, of the two salt preparations were the same.

No toxicity or reduced food intake was observed when control rats were injected with the matched Tris placebo.

Plasma pharmacokinetics

Fig. 1A shows that the plasma pharmacokinetics were different after i.p. injection of the two salts; slightly higher plasma levels of the Tris salt were achieved and maintained for longer than those of the sodium salt. This difference was observed at each point in time (Fig. 1A). Therefore the probability of difference at each point was assessed by a t-test, and these probabilities were combined as described by Fisher (1967). The difference between the two curves was significant ($P < 0.02$). In contrast, no difference was seen after i.a. injection (Fig. 1B). The following equations were fitted to the data of Fig. 1B by non-linear regression:

$$\begin{aligned} \text{Tris salt concentration in plasma (ng/ml)} &= (179 \pm 19) e^{-(0.0129 \pm 0.0050)t} \\ &+ (1579 \pm 23) e^{-(0.835 \pm 0.0449)t} \end{aligned}$$

$$\begin{aligned} \text{Sodium salt concentration} &= (156 \pm 8.4) e^{-(0.0107 \pm 0.0022)t} \\ &+ (1582 \pm 8.4) e^{-(0.628 \pm 0.0111)t} \end{aligned}$$

where t is the time (minutes) and \pm indicates the standard errors.

Discussion

The differences between the toxic effects of the two salts of 5-fluorouracil are dramatic, although our previous experiments showed that both salts caused diarrhoea and impairment of intestinal absorption (Gardner & Plumb, 1981). The significant difference between the plasma pharmacokinetics of the two salts after i.p. dosing suggests a possible explanation, and certainly reinforces our conclusion that the biological properties of the two salts are different.

Since different pharmacokinetics were observed after i.p. injection (Fig. 1A) but not after i.a. injection (Fig. 1B) it is plausible that the difference in Fig. 1A may have been due to more rapid uptake of the Tris salt from the peritoneal cavity, rather than to slower clearance from the blood. Although we cannot offer a definite explanation for this, it may be relevant that Tris base is a weaker base ($pK_a = 8.1$ at 25°C , Dawson, Elliott, Elliott & Jones, 1969) than sodium hydroxide. Hence, the Tris salt of 5-fluorouracil is likely to be less dissociated than the sodium salt, and thus may permeate some membranes more rapidly.

Although the higher plasma levels of the Tris salt than the sodium salt after i.p. injection might themselves account for the toxicity of the Tris salt, indirect evidence suggests that this is unlikely: the Tris salt, i.p., at a lower dosage (1.08 mmol/kg) was also associated with similar toxicity to that reported here. Although the rate constants for the overall removal of both salts from the plasma after i.a. injection were similar, it does not follow that the rate of uptake by every organ was necessarily

the same for both salts. Especially in the light of the apparently greater rate of uptake from the peritoneal cavity, it seems likely that some organs may accumulate the Tris salt faster than the sodium salt.

We do not know whether cardiotoxicity contributed to the mortality in our experiments; however, many authors have referred to acute cardiotoxicity apparently associated with 5-fluorouracil chemotherapy (Levillain, Ramona & Cluzan, 1974; Dent & McColl, 1975; Roth, Kolaric & Popovic, 1975; Lang-Stevenson, Mikhailidis & Gillet, 1977; Mikhailidis, Gillet & Lang-Stevenson, 1978; Pottage, Holt, Ludgate & Langlands, 1978; Soukop, McVie & Calman, 1978; Villani, Guindani & Pagnoni, 1979) although none stated which salt form was administered. We suggest that users of 5-fluorouracil might study their records retrospectively in order to see whether the incidence of adverse side effects, including cardiac ones, has changed since November 1978 when the sodium salt replaced the Tris salt on the U.K. market. It might then be possible to assess whether our observations at high dosage in rats are relevant to the clinical application of 5-fluorouracil.

Finally, our results emphasize the need to note full details about drug formulations in publications and, preferably, in clinical records.

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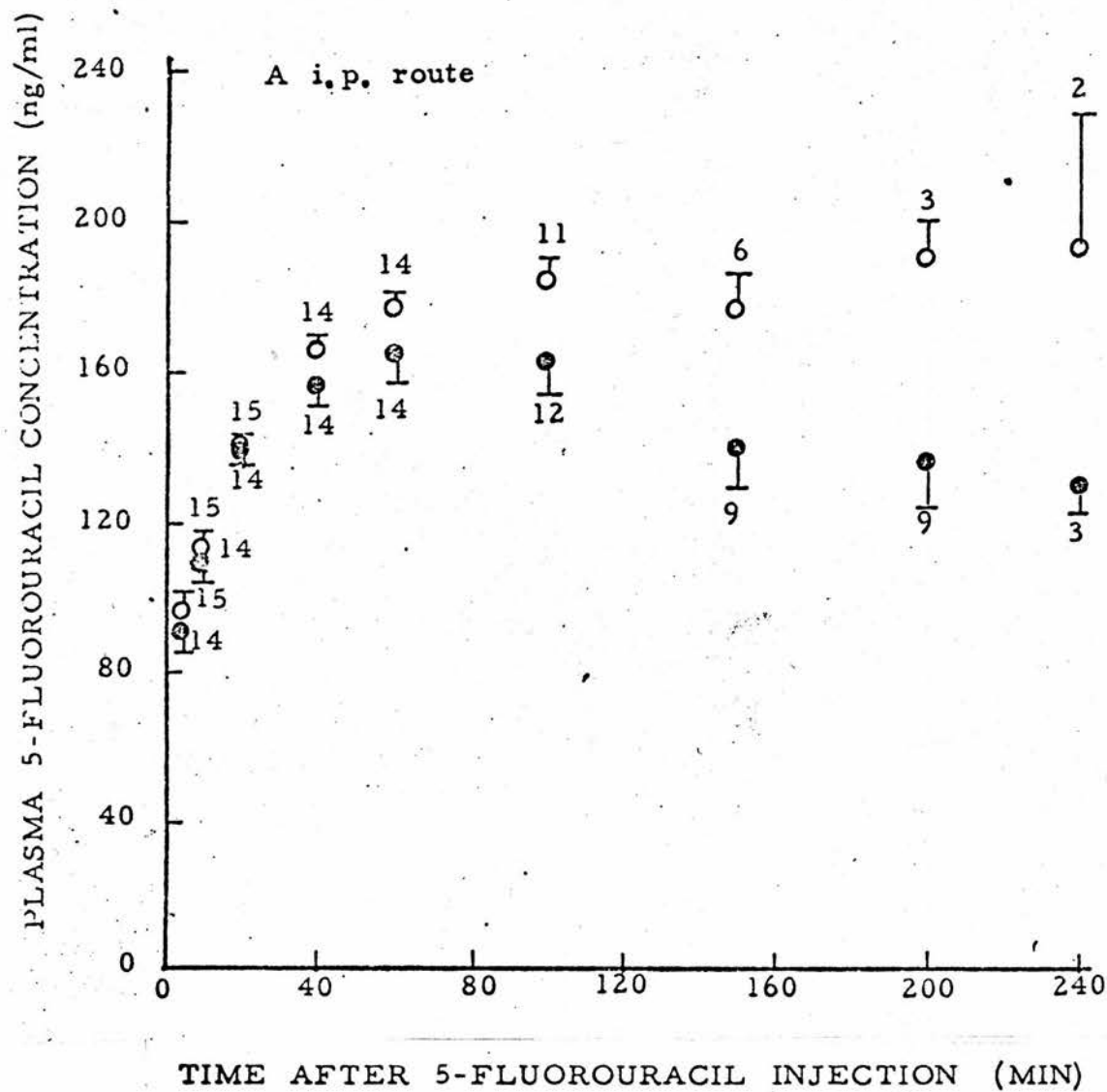
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Legend

Fig. 1. Plasma levels of 5-fluorouracil after (A) intra-peritoneal (i.p.) or (B) intra-arterial (i.a.) injection of either the sodium salt (●) or the Tris salt (○) in anaesthetized female rats. Values are means \pm S.E.M. and (A) the number of observations (n) is shown adjacent to each point, (B) each point is the mean of three or four observations after sodium or Tris salt injection respectively.



PLASMA 5-FLUOROURACIL CONCENTRATION (ng/ml)

